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Characterization and isolation of anti-HIV and latency reversal agents for the development of a herbal mixture of *Gnidia sericocephala*, *Senna italica*, *Sclerocarya birrea* and *Pentanisia prunelloides*

By

Babalwa Tembeni

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Summary

African traditional health practitioners play an important role in the lives of South Africans by providing locals with primary healthcare systems. Research shows that 80 percent of South Africans rely on traditional health practitioners for their healthcare needs. As a developing country with less resources, most people practice a different way of life that uses indigenous knowledge in curing diseases. A South African traditional health practitioner (THP) Nkabinde made claims that he uses a traditional medicine consisting of a mixture of four plants (*Sclerocarya birrea* bark, *Gnidia sericocephala* roots, *Senna italica* roots and *Pentanisia prunelloides* roots), for the treatment of human immunodeficiency virus (HIV) and other various diseases such as sexually transmitted diseases and opportunistic infections. The traditional health practitioner prepares a boiled water extract by combining the stem bark of *S. birrea*, dried ground roots of *S. italica*, *P. prunelloides* and *G. sericocephala*. After cooling the solid material is separated, and the decoction (water mixture) is used as a traditional medicine. The decoction is taken three times daily (4 tablespoons) under severe case of the disease the dosage can be taken up to 250 mL as a single dose. Nkabinde reported that his treatment does not have side effects and there is no known toxicity for his treatment. The full scientific evidence to support his claims was however lacking.

The plants *Sclerocarya birrea* bark and leaf, *G. sericocephala* roots, *S. italica* roots and *P. prunelloides* roots and the four plant mixture were extracted with dichloromethane : methanol (DCM : MeOH) (1:1 v/v). All the extracts were investigated for their *in vitro* anti-HIV activity at 25 and 2.5 µg/ml using a dual enhancement of cell infection to phenotypic resistance assay (DeCiPhR assay). The DCM : MeOH extract of *Gnidia sericocephala* showed very good inhibition activities at 25 and 2.5 µg/ml with minimum cytotoxicity levels. The DCM : MeOH extract of the mixture exhibited good anti-HIV activity mainly at 25 µg/ml. The anti-HIV activity of the mixture was attributed to the presence of the *G. sericocephala*, and the synergistic effect of the compounds present in the four plants extracts. Further screening of the

dichloromethane: methanol extract of the mixture and *G. sericocephala* showed significant anti-HIV activity in a dose dependant manner.

G. sericocephala roots were also extracted sequentially using hexane, dichloromethane, ethyl acetate and methanol. The extracts were further tested for their anti-HIV activity. The dichloromethane extract of *G. sericocephala* gave the best anti-HIV activity as it was the only extract to exhibit good anti-HIV activity in lower doses. The hexane extract only showed anti-HIV properties at higher concentrations and did not exhibit any anti-HIV properties at lower concentrations. The chemical profile of the DCM extract was obtained using an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) for the tentative identification of active compounds. The chemical profile showed a presence of daphnane diterpenoids, which are known for their anti-HIV activity.

In order to develop a pharmaceutically accepted extract, solvent mixtures of water and ethanol were used in different ratios to develop a standardised extract that can be developed as a traditional medicine for the treatment of HIV and concomitant latency reversal activity. The ethanol extract of *G. sericocephala* and 50% ethanol of the four-plant mixture gave the best anti-HIV activity with minimal cytotoxicity. After liquid-liquid partitioning between ethyl acetate and water, the ethyl acetate fraction of *G. sericocephala* showed increased HIV inhibition activity and latency reversal activity with minimal cytotoxicity. The 50% ethanol extract of the four-plant mixture gave the best anti-HIV and latency reversal activity.

The study reported for the first time daphnane diterpenoids present in *G. sericocephala* roots used as part of a traditional mixture in the treatment of HIV. The purification of these compounds was made successful by following multiple purification steps. A total of 30 microfractions obtained by fractionating the DCM extract using a high performance liquid chromatography (HPLC) resulted in five microfractions, labelled as 25, 26, 27, 28 and 29 being the only fractions to inhibit viral growth by $52.0 \pm 0.9\%$, $71.0 \pm 5.9\%$, $83.9 \pm 9.6\%$, $103 \pm 2.1\%$, $101 \pm 2.0\%$ and $91.6 \pm 2.8\%$, respectively. The chemical profiling of the combined

active microfractions using an ultra-performance liquid chromatography-quadrupole time of flight-tandem mass spectrometry (UPLC-QTOF-MS/MS) revealed a total of four daphnane diterpenoids (yuanhuacine A, yuanhuajine, gniditrin and yuanhuacine), which were linked to the anti-HIV activity observed in the microfractions and were prioritised for isolation and purification.

Two non-daphnane diterpenoids were isolated as biomarkers and later identified using one and two dimensional nuclear magnetic resonance (1D and 2D NMR) as lasioerin and lasiocephalin. The preparative purification of the active fractions resulted in isolation of five daphnane diterpenoids (yuanhuacine A, yuanhuacine, a cis 2''/3'' isomer of gnididin, yuanhuajine and gniditrin) with viral inhibition properties as well as latency reversal activity. The compound yuanhuacine A and the mixture of yuanhuacine and the cis 2''/3'' isomer of gnididin showed *in vitro* HIV replication inhibition by > 80% at 0.08 µg/ml. Furthermore, yuanhuacine A inhibited a subtype C virus replication in peripheral blood mononuclear cells (PBMC) with a half maximal effective concentration (EC₅₀) of 0.03 µM without cytotoxicity. The mixture of compounds, yuanhuacine and the cis 2''/3'' isomer of gnididin in an *in vitro* assay also reversed HIV latency by 16.7-fold lower concentrations than the control prostratin and also reversed HIV latency in primary helper T cells (CD4+ T cells) from combinatory anti-retroviral therapy (cART)-suppressed donors with HIV similar to the protein kinase C (PKC) activator prostratin but at 6.7-fold lower concentrations. These results suggests that *G. sericocephala*, yuanhuacine A and the mixture of yuanhuacine and a cis 2''/3'' isomer of gnididin act as anti-HIV agents to improve cART efficacy and support HIV cure efforts in resource-limited regions.

The study reported for the first time the semi-pilot scale development of the ethyl acetate fraction of *G. sericocephala* roots using good manufacturing practices (GMP) guidelines outlined for the development of traditional medicine. The developed product passed all quality control analysis requirements which included physical, chemical, heavy metal, total solids, aflatoxins, microbial, and pesticides specifications. The fraction also showed good *in vitro* HIV

replication inhibition with a half maximal inhibitory concentration $IC_{50} = 0.05 \mu\text{g/ml}$ and *ex-vivo* HIV replication inhibition with an $IC_{50} = 0.06 \mu\text{g/ml}$. The fraction also showed retained latency reversal activity with an $IC_{50} = 0.3 \mu\text{g/ml}$. These results showed a retained activity by the fraction from laboratory scale to semi-pilot scale suggesting that the extraction in pilot scale production was a success.

The UPLC-MS profile of the ethyl acetate fraction revealed a presence of 11 compounds, of which four belonged to the class of daphnane diterpenoids. These compounds (the daphnane diterpenoids) were attributed to the anti-HIV and latency reversal activity. The latency reversal activity of the ethyl acetate fraction of *G.sericocephala* was found to be comparable to prostratin. The quantitative analysis of yuanhuacine A in the ethyl acetate fraction of *G. sericocephala* using a five-point concentration in triplicate suggested that the fraction constitutes of 1.5 % of yuanhuacine A.

The 50% ethanol extract of the four-plant mixture was also developed for the first time in a semi-pilot scale following GMP guidelines. The extract passed all quality control specifications such as physical, chemical, heavy metal, aflatoxins, pesticides, total solids, and microbial specifications. The semi-pilot scale extraction of the 50% ethanol of the four-plant mixture however posed some challenges as more advanced drying techniques were required to completely remove the solvents in the extract. Prior to the extract being exposed to high temperatures its chemical fingerprint using UPLC-qToF-MS/MS showed the presence of daphnane diterpenoids, however after the extract was exposed to high temperatures during oven drying the daphnane diterpenoids were not detected. The absence of daphnane diterpenoids in the extract was attributed to the loss of the anti-HIV and latency reversal activity of the extract in lower test concentration when compared to the laboratory developed product. The oven drying process was established as not being an ideal method of drying these products and a more suitable drying method that requires lower temperatures to avoid the loss of the active ingredient is required for this extract. Due to this, yuanhuacine A could not be quantified in the over dried extract.

Moreover, the dual activity inhibition of HIV replication and HIV latency reversal shown by these compounds suggested further investigation on the mode of action of the compounds and the extracts in additional cell and animal models. As South Africa continues to have a high number of people living with HIV showing resistance to available antiretroviral therapy with limited access to existing cART. The compounds and extracts of *G. sericocephala* could act as useful drug leads toward additional therapies for managing HIV infection and acquired immune deficiency diseases (AIDS) pathogenesis to supplement cART.

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Abbreviations

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^1H NMR	Proton NMR
AIDS	Acquired immunodeficiency syndrome
AZT	azidothymidine
BPI	Base peak ion
cGMP	Current Good Manufacturing Practices
COSY	Correlation spectroscopy
DCM	Dichloromethane
DeCiPhR assay	Dual enhancement of cell infection to phenotype resistance assay
DEPT	Distortionless enhancement by polarisation transfer
ELSD	evaporative light scattering detector
EtOAc	Ethyl acetate
ESI	Electron spray ionisation
FA	Formic acid
GFP expression	Green Fluorescent Protein expression
GMP	Good Manufacturing Practices
<i>G. sericocephala</i>	<i>Gnidia sericocephala</i>
HDACi	histone deacetylase inhibitor
HIV	Human immunodeficiency virus
HPLC-QTOF-MS	High-performance-liquid chromatography/quadrupole-time-offlight mass spectrometry
HPLC-MS-SPE	High-performance-liquid chromatography-mass spectrometry-solid phase extraction
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
hERG	human ether-a-go-go
IC ₅₀	The half maximal inhibitory concentration
IC ₉₀	The concentration that will inhibit 90% of the virions.
iFit	Isotopic fit value
LRA	latency-reversing agent

MeOH	Methanol
min	Minute
ml	millilitre
MS	Mass spectrophotometry
<i>m/z</i>	Mass to charge ratio
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PBMCs	Human peripheral mononuclear cells
ppm	Parts per million
<i>P. prunelloides</i>	<i>Pentania prunelloides</i>
Prep HPLC-MS	Preparatory high-performance- liquid- chromatography coupled to a Mass spectrometer
PKC	protein kinase C
<i>S. birrea</i>	<i>Sclerocarya birrea</i>
SI	Selectivity index
<i>S. italica</i>	<i>Senna italica</i>
TLC	Thin layer chromatography
µg/ml	Microgram per millilitre
µM	Micromolar

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Chapter one

General introduction

1.1 Medicinal plants contribution to treating diseases

Throughout history, humans have exploited plants as sources of food and medicine.^[1] Therefore, plants have been a subject of research in an attempt to understand their nutritional and medicinal properties.^[2] As a source of medicine, plants are used as decoctions, whereby the whole plant is boiled in water and taken as tea or its vapour inhaled to treat many ailments.^[3] In the early 19th century researchers discovered that single pure compounds can be isolated from these plants.^[4] This led to the first isolation of an analgesic drug morphine **(1)** from *Papaver somniferum* L. (opium poppy), an antimalarial drug quinine **(2)** from *Cinchona succirubra*, digitoxin **(3)** a cardiotonic glycoside from *Digitalis purpurea* L (foxglove) and a parasympatholytic alkaloid atropine **(4)** from *Atropa belladonna* shown in Figure 1.^[4,5] The isolation of these drugs contributed immensely in the production of more potent analogues with a vast array of pharmacological value when compared to the parent compounds.^[6] This is evident as available pharmaceutical drugs on the market have a backbone in natural product.^[7] The production of natural product analogues is done to either increase potency or reduce toxicity.^[7] Some analogues were synthesised to produce drugs for different ailments. ^[7]

Plants biosynthesise secondary metabolites as defence mechanism in the fight against predators such as pollinating animals, bacteria, and fungi.^[8] These compounds are said to be specific to a certain plant or genus and are known as secondary metabolites.^[9-10] Secondary metabolites can be categorised into three main groups and these include alkaloids, phenolics and terpenes.^[8] Alkaloids are commonly known to contain nitrogen in a heterocyclic ring and are derivatives of amino acids.^[11] Alkaloids represent a large diverse group of compounds and these include codeine **(5)**, cocaine **(6)** and taxol **(7)** (Figure 1).^[12]

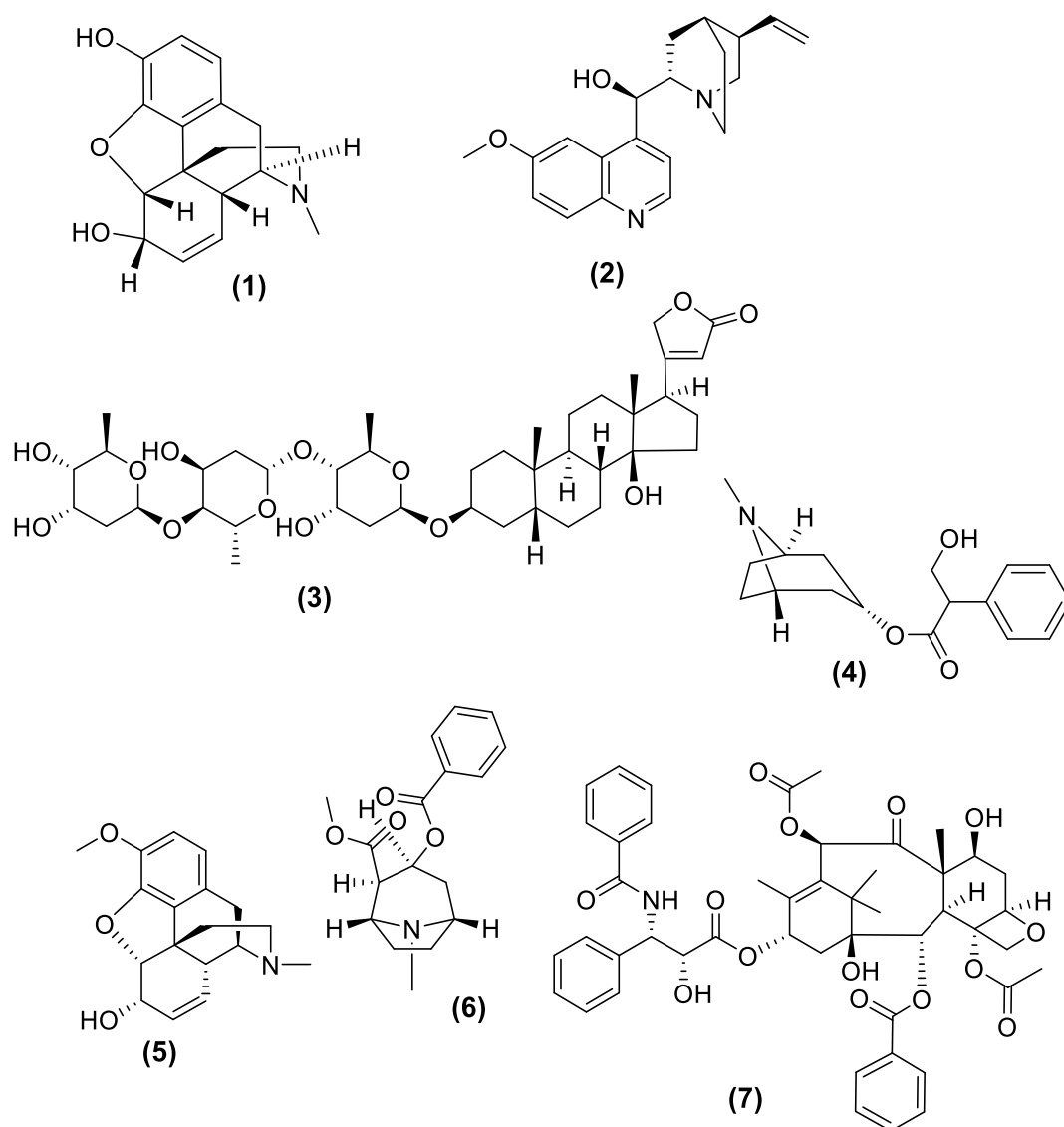


Figure 1: Chemical structure of morphine **(1)**, quinine **(2)**, digitoxin **(3)**, atropine **(4)**, codeine **(5)**, cocaine **(6)** and taxol **(7)**.

Phenols are a class of compounds that contain a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group.^[13] Both these classes have been shown to possess medicinal properties. Terpenes and their oxygenated derivatives have also been shown to possess medicinal properties.^[14] The chemical diversity of plant based natural product compounds has provided human beings with unlimited pharmacological, cosmeceutical and nutraceutical opportunities especially in the discovery of new drugs.^[14]

These compounds have been isolated as single or pure compounds, as a result of the advancement of analytical techniques used in the identification and purification of these compounds. This breakthrough led to an increase and demand for natural products based compounds.^[15] It is also understood that a single compound allows for the understanding of specific interactions they have with biological macromolecules found in the body to understand the mechanism of action. ^[7,13,14,16,17]

1.2 Medicinal plants of South Africa

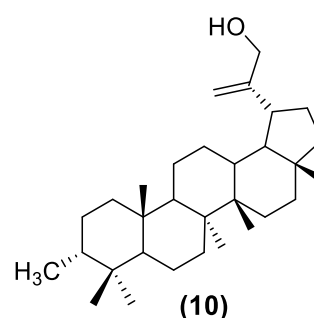
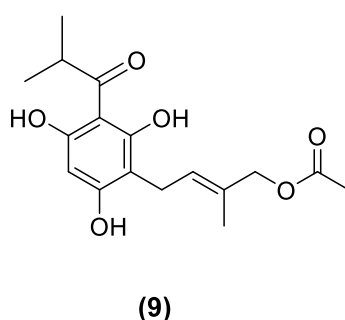
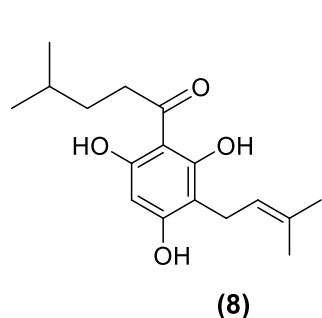
South Africa has a rich biodiversity, and it houses approximately 30 000 plant species. which accounts for almost 10 percent of the world's plant diversity.^[18] The country has over 200 000 traditional health practitioners who make use of these plants to manage and cure different ailments such as HIV.^[19] About 80 percent of the population, particularly, those who live in the rural areas consume approximately 20 000 tons of medicinal plants on an annual basis as a supplement to orthodox medicine.^[20,21] The medicinal properties of South African plants are being investigated by local and international researchers to obtain compounds with pharmacological value.^[6,22]

Numerous studies investigated the chemical properties and the activity of compounds on various diseases and infections such as arthritis, cancer, diabetes, hypertension flu, HIV and many more.^[6,19,21–25] Studies on South Africa's famous *Helichrysum caespitium*, traditionally used for its medicinal properties in the treatment of many ailments attributed the pharmacological properties of this plant to the anti-bacterial compound caespitin (**8**) and the anti- tuberculosis and anti-fungal compound caespitate (**9**) (Figure 2) present in the plant amongst other compounds.^[26,27]

Elaeodendron transvaalense a famous plant amongst South Africans, is widely used traditionally in the treatment of ulcers, arthritis, respiratory tract infections, cancer, fever and diarrhoea.^[21,28,29] Phytochemical investigations on the species revealed the presence of lupane triterpenoids which are known for their anti-cancer properties. The triterpenoids include

lup-20(30)-ene-3 α -29-diol (**10**), 6 β -hydroxylup-20(29)-ene-3-one (**11**), 30-hydroxylup-20(29)-ene-3-one (**12**). The anti-microbial properties of the plant were reported to be due to the presence of 4'-O-methyl epigallocatechin (**13**) (Figure 2). [27,30]

Glycyrrhiza glabra found in some parts of Africa, Asia and Europe is also found in South Africa. The plant is traditionally used for its medicinal properties that include anti-viral, anti-ulcer, anti-diabetic amongst others. In South Africa the plant is also used in a cocktail consisting of other medicinal plants for the treatment of TB. The medicinal properties of the plant has been investigated extensively all over the world. The structures of compounds such as glycyrrhizin (**14**) with known anti-viral, anti-ulcer and anti-inflammatory activities which have been isolated from *G. glabra*. Compounds such as liquiritin (**15**), glycyrrhetic acid (**16**), liquiritin apioside (**17**) and glabridin (**18**) are shown in Figure 2. The compounds have also been identified and isolated from the plant. The phytochemical studies on *G. glabra* provides scientific data to substantiate the traditional use of the plant.[21]



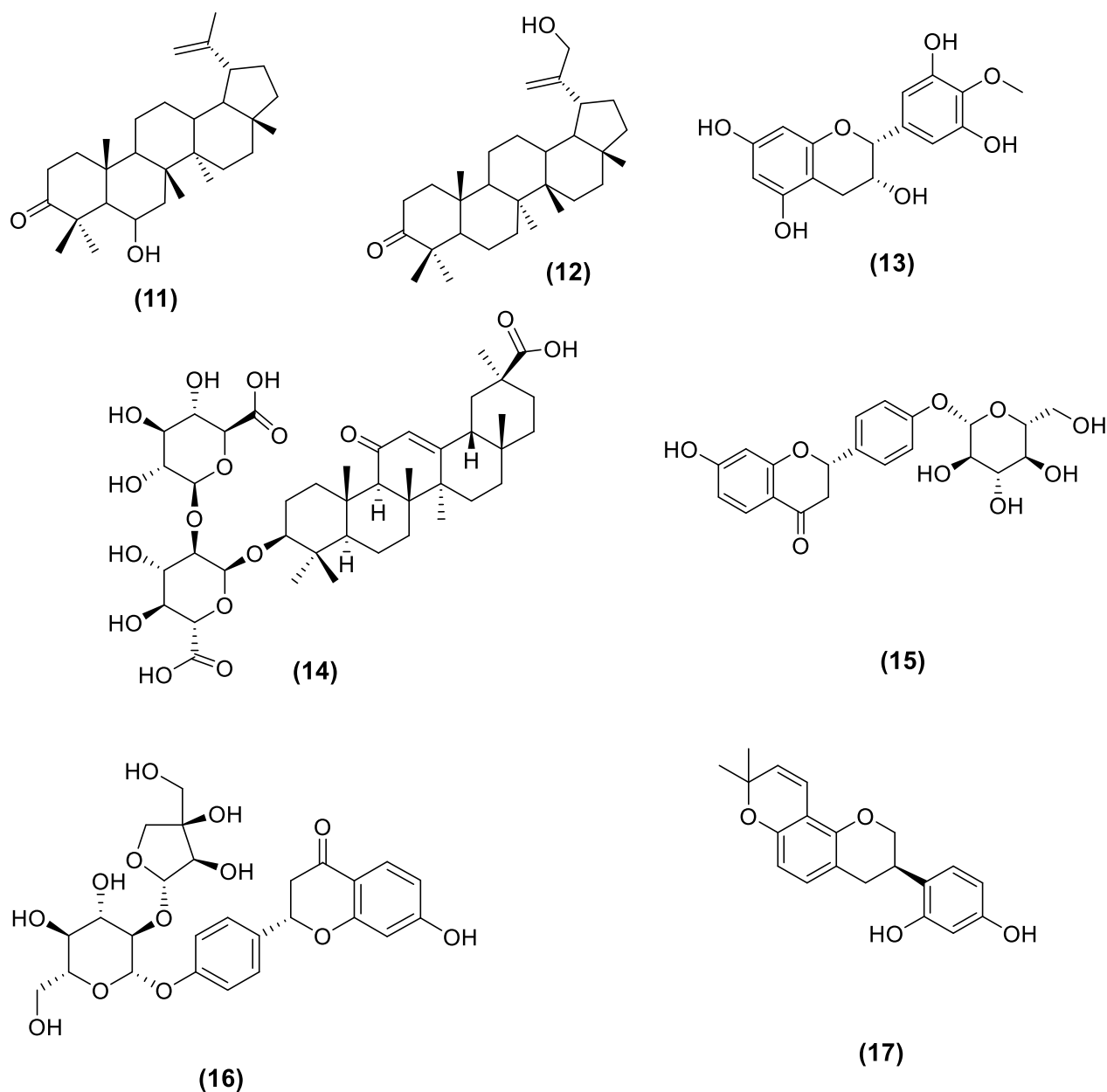


Figure 2: Structure of ceaspitin (**8**), caespitate (**9**), lup-20(30)-ene-3 α -29-diol (**10**), 6 β hydroxylup-20(29)-ene-3-one (**11**), 30-hydroxylup-20(29)-ene-3-one (**12**), 4'-O-methyl epigallocatechin (**13**), glycyrrhizin (**14**), liquiritin (**15**), liquiritin apioside (**16**) and glabridin (**17**).

1.2.1 The role of indigenous knowledge systems

African traditional health practitioners play an important role in the lives of South Africans by providing locals with primary healthcare systems.^[18,31,32] Research shows that 80 percent of South Africans rely on traditional health practitioners for their healthcare needs. As a

developing country with less resources, most people practice a different way of life that uses indigenous knowledge in curing diseases.^[18] This knowledge has been passed on verbally from generations to generations. Hence, it is said that indigenous knowledge systems have an important contribution in the socioeconomic growth and sustainability in the country.^[33] This contribution can be seen from the numerous traditional medicines that are currently on the market and being used by traditional health practitioners.^[31]

Traditional health practitioners use various methods of healing such as, herbalism, divination and spiritualism as a corner stone in the treatment of many ailments.^[31] Traditional health practitioners have been reported to use a holistic approach that treats the disease and the mental well-being of the patient.^[34] In addition, access to main stream healthcare systems in the rural areas is limited. Hence, people prefer traditional health practitioners who are easily accessible to them at a cheaper price.^[35]

Most importantly, the high uptake of traditional medicine is driven by common misconceptions that natural products are free from toxicity and side effects.^[36] In addition, access to main stream healthcare systems in the rural areas is limited. Despite the use of South African medicinal plants by many people, their medicinal properties are still poorly recorded. Hence, the need to further investigate all South African plants for their efficacy and toxicity.^[31,37]

1.2.2 Commercial use of traditional medicine

South Africa has approximately 3000 medicinal plants with reported pharmacological value.^[12] The country only has about 40 of its indigenous plants in formal and informal markets.^[38] These plants include *Aloe ferox* also known as cape aloe, which has extensive commercial uses in cosmeceuticals because of its rich content in amino acids, vitamins, and trace elements.^[22] *Aspalathus linearis* also known as rooibos tea is commercialised as a herbal tea due to its rich content of iso-vixentin **(18)**, vixetin **(19)**, aspalathin **(20)**, nothofagin **(21)**, orientin **(22)** and quercitrin **(23)** (Figure 3).^[22] The herbal tea is said to have a variety of health benefits such as anti-oxidative properties, anti-eczema, anti-ageing and anti-plasmodic properties.^[22]

Pelargonium sidoides is a licenced respiratory tract infection remedy also known as Umckaloabo and is a rich source of prodelphinidins, methoxy coumarins and proanthocyanidin.^[22] *Siphonochilus aethiopicus* also known as African ginger is commercialised for its medicinal properties in the treatment of colds, asthma and influenza with (4aR,8aR,9aS)-3,8a-Dimethyl-4a,8a,9,9a-tetrahydronaphtho[2,3-b]furan-2,8(4H,5H)-dione, **(24)** and siphonochilone **(25)** as biomarkers of the plant (Figure 3).^[39] *Sclerocarrya birrea* is a commercially valuable plant of South Africa. All the plant parts of *Sclerocarrya birrea* play a crucial role in its commercialization, as they are either of pharmacological, nutraceutical and cosmeceutical value. The plant is commercialised as gels, creams, juices, jelly and jams.^[8,22,40]

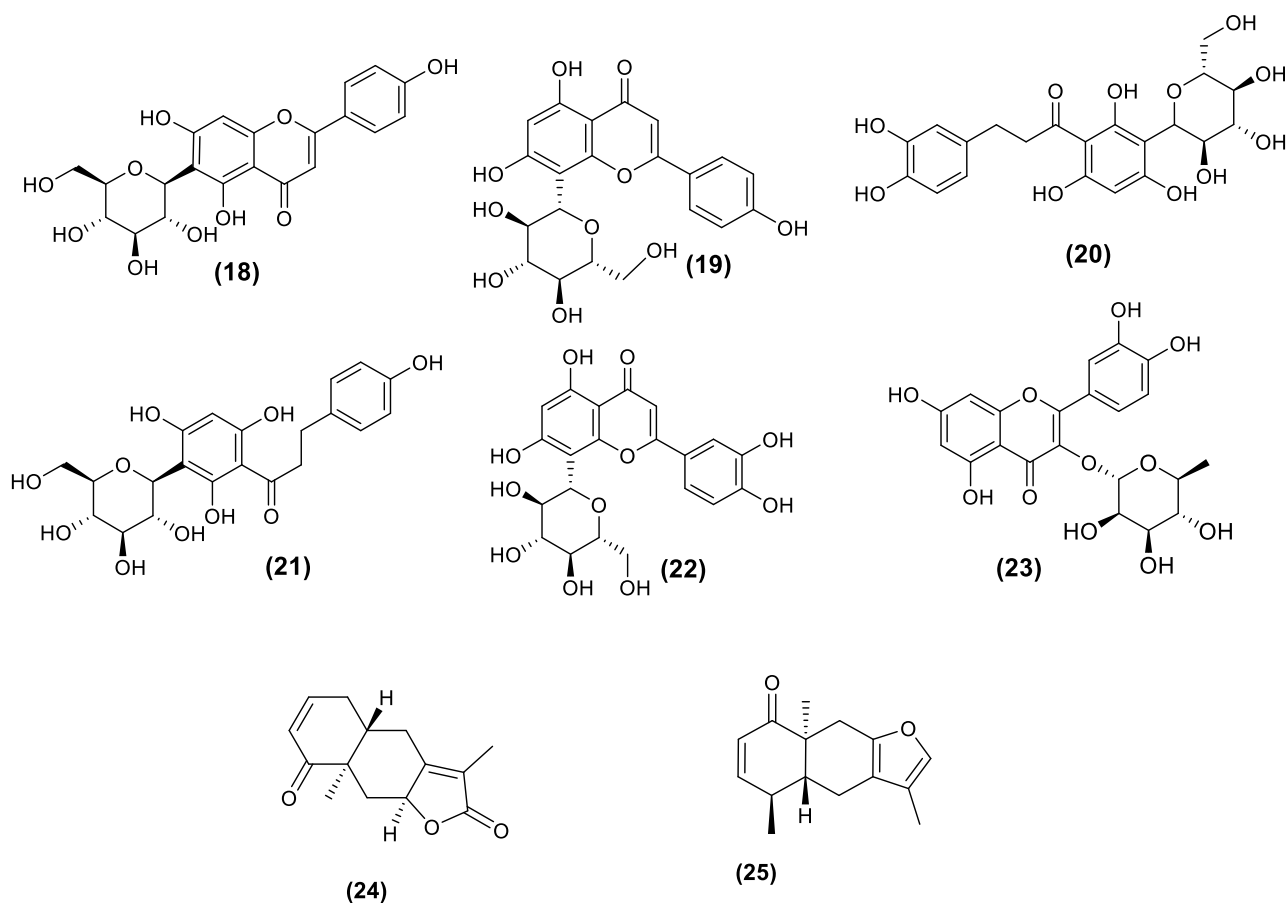


Figure 3: Structures of iso-vixentin **(18)**, vixetin **(19)**, aspalathin **(20)**, nothofagin **(21)**, orientin **(22)**, quercitrin **(23)**, (4aR,8aR,9aS)-3,8a-dimethyl-4a,8a,9,9a-tetrahydronaphtho[2,3-b]furan-2,8(4H,5H)-dione **(24)** and siphonochilone **(25)**

1.3 The Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) (Figure 4) is a single stranded ribonucleic acid (RNA) virus that infiltrates the human body for survival.^[41] It is an enveloped retrovirus with a diameter of 100-120 nm that consists of a phospholipid bilayer with spikes of glycoprotein gp41 and gp120.^[42] The gp120 of HIV binds to the cell surface of CD4 + T-cells molecules and initiates fusion with the cell, involving gp41.^[43] Inside the envelope is a nucleocapsid, which surrounds a central core protein p24 (Figure 4).^[44] The single stranded RNA is firmly bound to the nucleocapsid proteins, p7 and p9.^[44] Enzymes needed for the development of the virions such as reverse transcriptase, proteases, ribonuclease and integrase (Figure 4), form part of this core and are responsible for the development of AIDS.^[44,45]

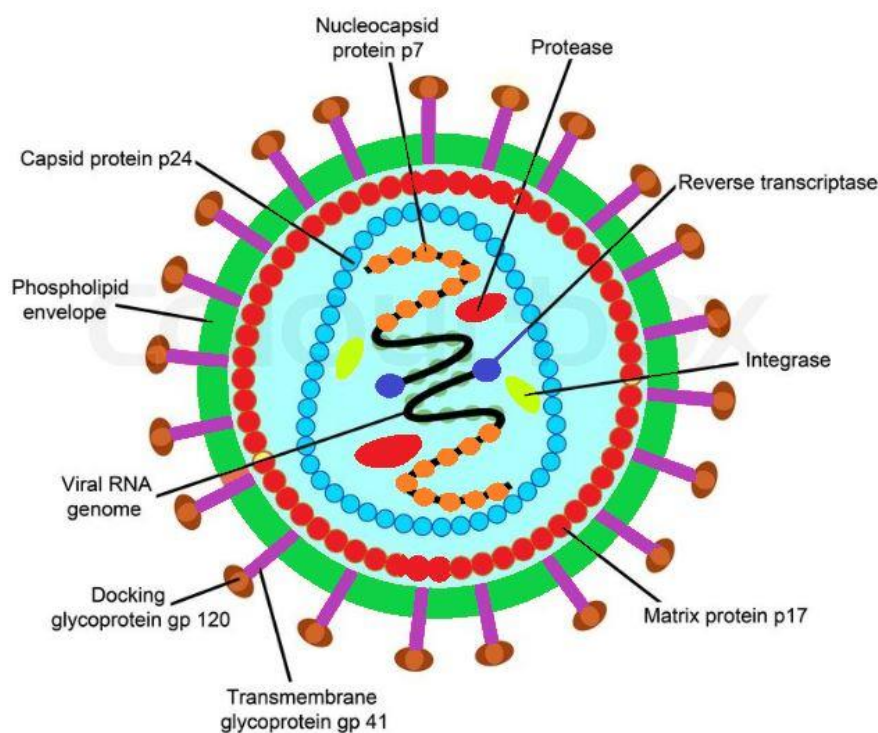


Figure 4: Structure of HIV. ^[19]

1.3.1 HIV replication cycle

Upon entry gp120 binds with CD4⁺ T cells and co-receptors CCR4 to allow the unfolding and fusion of the gp41 into the cytoplasm of the cell to release the viral capsid with three key enzymes that it uses to replicate.^[46] The viral capsid disintegrates and the enzyme reverse transcriptase (RT) converts the viral RNA into a viral DNA and the process is known as reverse transcription.^[47] This replication cycle often gives rise to viral mutations that offer drug-resistance or allow the virus to evade the body's immune system due to the highly error-prone nature of reverse transcriptase.^[43]

HIV integration is immediately initiated whereby, the proviral DNA is integrated into DNA of the host cell by enzyme integrase.^[48] After this stage, HIV replicates by making long chains of proteins that act as building blocks for more HIV.^[49] The new viral proteins and RNA are transported into the surface of the cell and assemble into immature HIV to initiate budding.^[42] The newly formed immature virions bud out of the host cell and release the enzyme protease. Enzyme protease converts the immature virions into mature infectious HIV by breaking up the long protein chains in the immature virions.^[50]

The replication steps of the virus have been used to develop anti-HIV drugs that will inhibit specific sites and enzymes.^[41] Examples of such drugs are fusion inhibitors, reverse transcriptase inhibitors, non-reverse transcriptase inhibitors, protease inhibitors.^[50,51]

1.3.2 HIV inhibitors and their functions

The complex nature of the enzymatic stages of the HIV virus has led to the design and development of anti-retroviral drugs.^[52–54] The anti-retroviral drugs target one or more stages in the HIV replication cycle to prevent the progression of HIV to AIDS.^[44,47] It is of great importance to take note that all the developed anti-retroviral drugs on the market are not plant derived but are chemically synthesised.^[55]

Zidovudine (**26**) also known as azidothymidine (AZT) is the first nucleoside reverse transcriptase inhibitor to be approved by the Food and drug administration (FDA) for the treatment of HIV (Figure 5).^[56] This drug led to the development of other anti-retroviral drugs such as efavirenz (**27**), nevirapine (**28**), a non-nucleoside reverse transcriptase inhibitors (NNRTIs) that blocks the transcription stage of HIV (Figure 5).^[56] These NNRTIs prevent the enzyme reverse transcriptase from converting the viral RNA into viral DNA.^[57] Other anti-retroviral drugs include nucleoside reverse transcriptase inhibitors (NRTIs).^[57] These drugs also prevent the enzyme reverse transcriptase from transcribing viral RNA into viral DNA and they include stavudine (**29**), tenofovir (**30**) and lamivudine (**31**) (Figure 5).^[41]

All the enzymes in the HIV life cycle have been targeted by one or two types of inhibitors.^[58] Protease inhibitors selectively bind to the enzyme protease.^[42] The inhibitors block the proteolytic cleavage of protein precursors responsible for the production of infectious virions and they include ritonavir (**32**) and darunavir (**33**) (Figure 5), amongst others.^[41] Entry and fusion inhibitors prevent the binding and fusion of the virus.^[41] Entry and fusion inhibitors prevent the viral entry into host cell by binding to the glycoproteins of the virus, or the fusion of the gp120 to the CD4⁺ T-cells such as maraviroc (**34**) and integrase inhibitors are responsible for the prevention of the action of integrase they include raltegravir (**35**) (Figure 5).^[56]

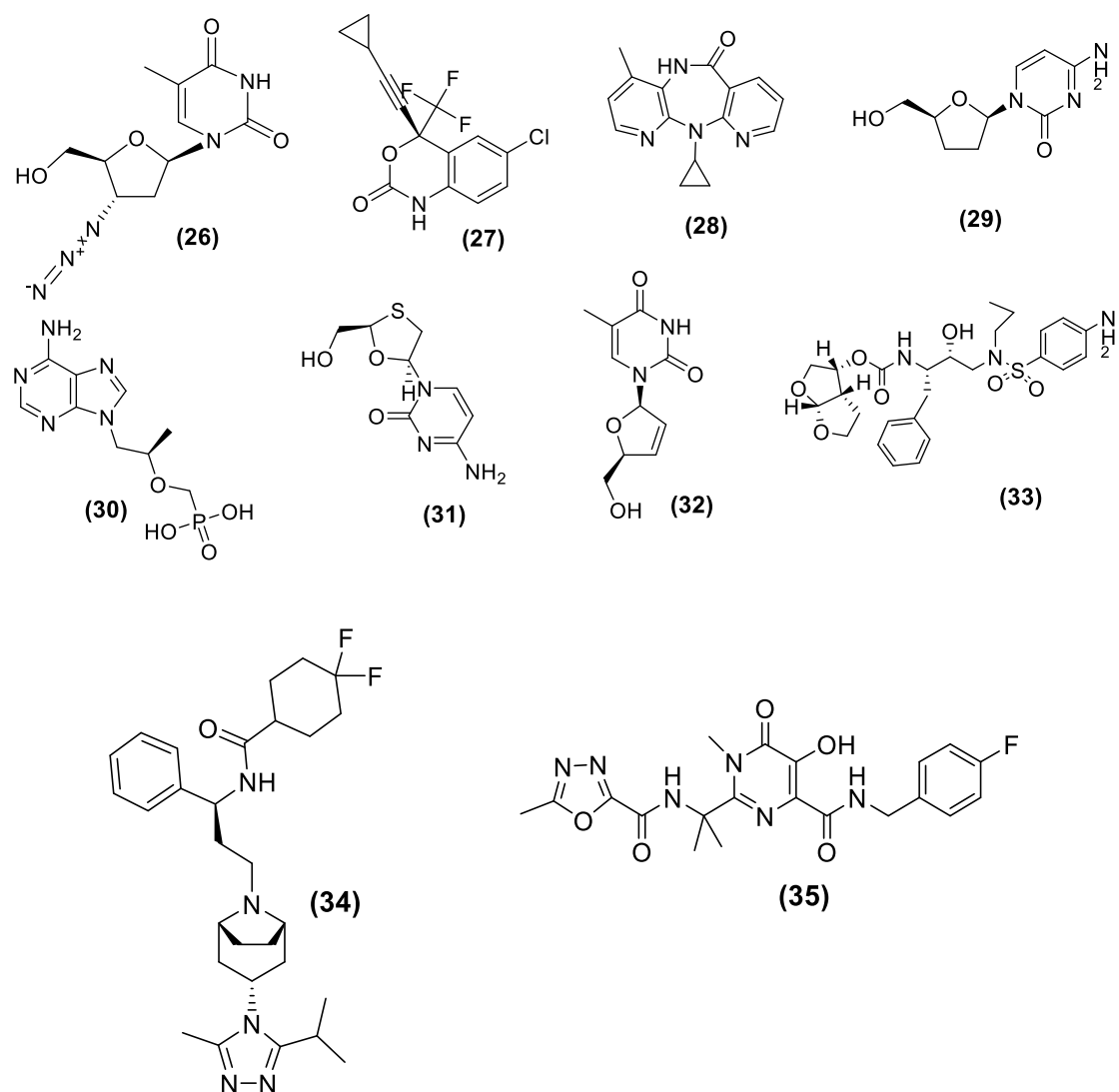


Figure 5: Structure of zidovudine (26), efavirenz (27), nevirapine (28), zalcitabine (29) tenofovir (30), lamivudine (31) stavudine (32), darunavir (33), maraviroc (34), and raltegravir (35)

1.3.3 HIV in South Africa

The Joint United Nations Programme on HIV/AIDS (UNAIDS) reported South Africa to have an estimated 7.6 million people living with HIV.^[33,59,60] Despite the introduction of highly active anti-retroviral therapy (HAART) in 2003 and the implementation of the universal test and treat programme in 2016, the country still accounts for the worlds twenty percent of people living with HIV and twenty percent of new infections.^[33,60] These new infections were reported by the South African Human Sciences Research Council (HSRC) to be caused by the large number

of individuals defaulting on treatment, slow switching of patients with drug resistance variants to other regimes, failing to monitor patients' CD4 count levels as well as alcohol and drug abuse (Figure 6).^[33,60–62] It is also an undeniable fact that the introduction of a HAART also decreased mortality rates drastically as it assisted in suppressing viral infections and boosting the immune system among individuals who were loyal to their treatment (Figure 6).^[33,60–62]

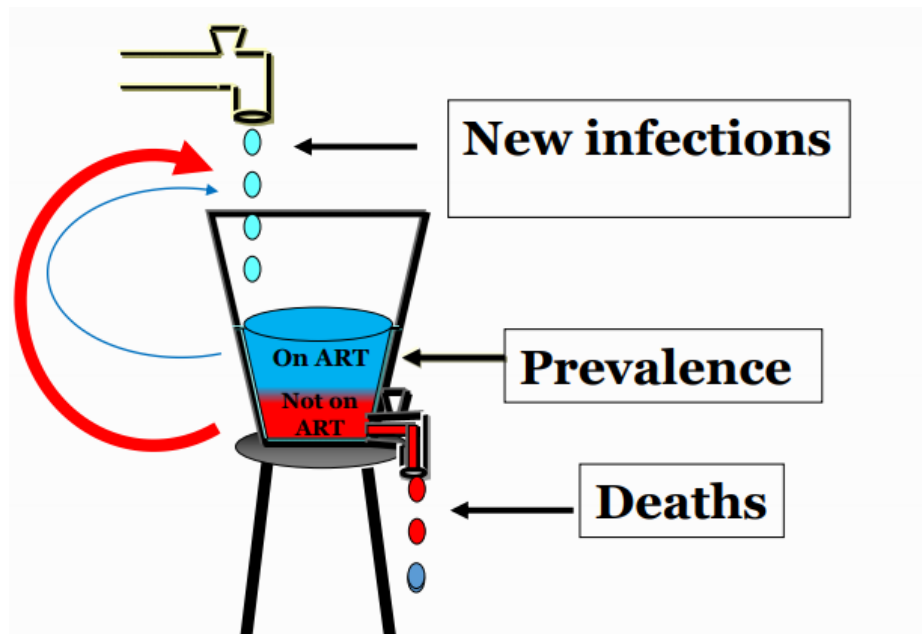


Figure 6: Contextualizing the HIV epidemic in South Africa.^[63]

1.3.4 Challenges faced with current treatment

The introduction of a highly active anti-retroviral therapy (HAART) in South Africa significantly reduced the number of fatalities due to HIV and AIDS.^[33,64] HAART as a very effective method of fighting HIV comes with challenges faced by its users.^[18,25] These challenges include drug resistance due to viral mutations.^[65] Other adverse effects include renal failure, anaemia, central nervous system disorders, hepatitis amongst others. The greatest setback of the therapy would be its inability to eradicate the virus in the body completely.^[51] This is due to viral particles that are not responding to HAART as they are hidden in human cells that are not metabolically active in the body.^[47] Latent cells in the body comprises of a HAART-persistent latent reservoir with long half-lives.^[47,66,67] The latent reservoirs are the main source

of new viral production with viral mutations that lead to drug resistance and eventually AIDS when the cells are activated.^[51]

Latency in viral cells is a very dangerous stage as an individual who is undergoing HAART will have an increase in CD4⁺ T-cells and a decrease in viral load.^[68] The moment the latent cells get activated due to misuse of the therapy there will be a drastic increase in viral load and decrease in CD4⁺ T-cells. This then leads to AIDs and eventually death.^[51,68] Given the foregoing, there is a need to have drugs that will not only suppress the virus but eradicate it completely in the body by activating the latent reservoirs in the body.^[69]

Some natural products compounds have been investigated for their ability to activate the latent cells.^[68] These compounds include a class of compounds known as phorbol esters and bryostatin-1 which is a macrolide lactone. Some phorbol esters macrolide lactones are known for their ability to re-activate latent reservoirs of HIV by modulating the protein kinase c (PKC) pathway which induces latent HIV1 expression.^[69,58,63]

1.4 Natural products as a treatment for HIV

The plants ability to biosynthesise compounds as a means of protection against many factors has benefitted mankind with a tool to fight disease causing viruses.^[4] This ability has been given full consideration in the treatment of HIV after the emergence of new viral strains that show resistance to available anti-retroviral drugs.^[36] As a source of new drug leads, plants have shown potential to possess compounds with anti-HIV properties.^[50] This is shown by the number of plants that have been biologically screened for their anti-HIV properties.^[54] Most of these plants contain compounds that possess remarkable anti-HIV properties.^[68] Natural product compounds with anti-HIV properties have also been proven to be a source of new mode of action in the treatment of HIV by targeting various replication processes in the HIV.^[52] This is because they have diverse and complex compounds.^[68,70]

1.4.1 Role of natural products in HIV treatment

Amongst the early natural product drugs for the treatment of HIV are calanolides A (**36**) and B (**37**) from *Calophyllum lanigerum* (Figure 7). These compounds were found to possess non-nucleoside reverse transcriptase inhibition activities but due to the scarcity of the plant, the drugs had to be synthesised.^[4,36] Betulinic acid (**38**) isolated from *Syzygium claviflorum* is another anti-HIV compound from a plant and it has the ability to promote the release of non-infectious viral particles (Figure 8). The anti-HIV activity of betulinic acid was improved by synthesising betulinic analogues by esterification at C-3 hydroxyl resulted in more potent compounds with tremendously improved therapeutic index values.^[36,70] Most plant based natural products with anti-HIV properties belong to different classes of compounds such as alkaloids, terpenes, coumarins and phenolics.^[71,38]

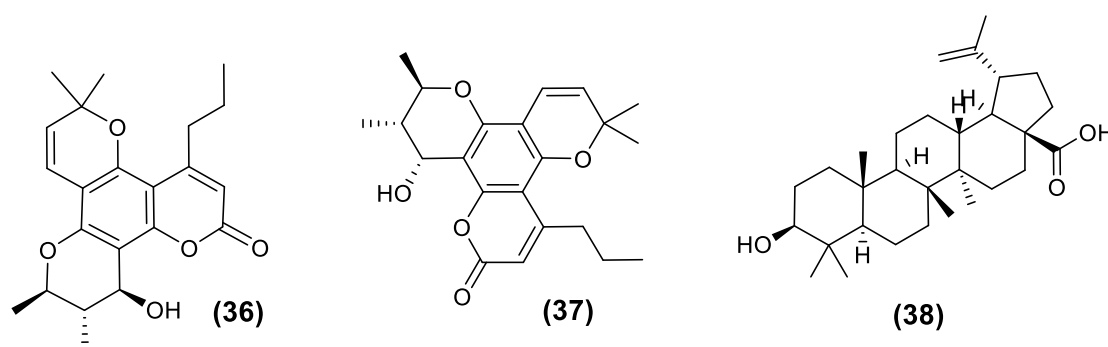


Figure 7: Structures of calanolide A (**36**), calanolide B (**37**), and betulinic acid (**38**)

1.4.2 Alkaloids as anti-HIV agents

Alkaloids are plant based compounds, they are known to be basic, contain nitrogen atom/s in a heterocyclic ring (Figure 8).^[73] These compounds exhibit numerous physiological and pharmacological mechanisms. Alkaloids are mostly derived from amino acids and their classification is based on their structure. Several alkaloids have been reported to exhibit anti-HIV properties.^[19,36] Michellamine B (**39**) from *Ancistrocladus korupensis* was proven to have reverse transcriptase activity, these compounds also have the ability to block cell fusion and

syncytium formation (Figure 8).^[74] Castanospermine (**40**) an alkaloid from *Castanospermum australe* showed anti-HIV replication properties and also blocks the formation of syncytia formed after glycoproteins gp120 fusion (Figure 8).^[75] A reverse transcriptase inhibitor nitidine (**41**) from *Toddalia asiatica* also forms part of alkaloids that exhibit good anti-HIV properties (Figure 8).^[36] A monoterpenoid-tetrahydro isoquinoline compound emetine (**42**) from *Psychotria ipecacuanha* and corydine (**43**) from *Croton echinocarpus* possess anti-HIV properties (Figure 8). These compounds have the ability to block the reverse transcriptase, thus inhibiting HIV replication.^[76,57]

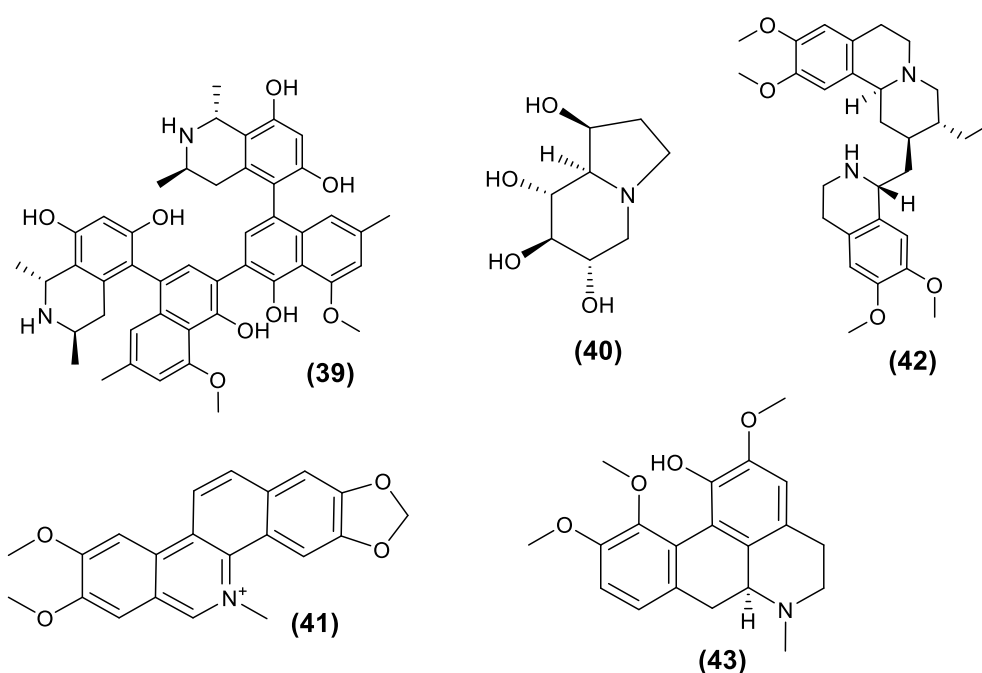


Figure 8: Structure of michellamine B (**39**), castanospermine (**40**), nitidine (**41**), emetine (**42**), and corydine (**43**)

1.4.3 Terpenes as anti-HIV agents

Terpenes and their oxygenated derivatives are a structurally diverse group of natural products formed by fusion of isoprene unit monomers in plants (Figure 9). This class of plant secondary metabolites are grouped according to the number of isoprene units or numbers of carbon in their skeletal structure e.g. monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes.^[36] Different subclasses of terpenes have been shown to exhibit antiretroviral

properties with different modes of action. Triterpenoids such as masilinic acid (**44**) and oleanolic acid (**45**) have been shown to possess anti-HIV properties. Maslinic acid is a triterpenoid isolated from *Geum japonicum* and it showed inhibition activities against the enzyme protease (Figure 9). Oleanolic acid isolated from *Xanthoceras sorbifolia* inhibits the viral replication. Diterpenes and their oxygenated derivatives have also been shown to possess antiretroviral activities targeting various stages in the viral cycle.^[4,36,77] Prostratin (**46**) a phorbol ester diterpenoids first isolated from *Pimelea prostrata* exhibits good antiretroviral activities (Figure 9). This compound acts as a protein kinase C activator and also reduces the formation of latent reservoirs (dormant HIV cells).^[36] Ingenol mebutate (**47**) from *Euphorbia peplus* is another diterpenoid ester that has the ability to reactivate HIV latent reservoirs (Figure 9).^[16,48,73]

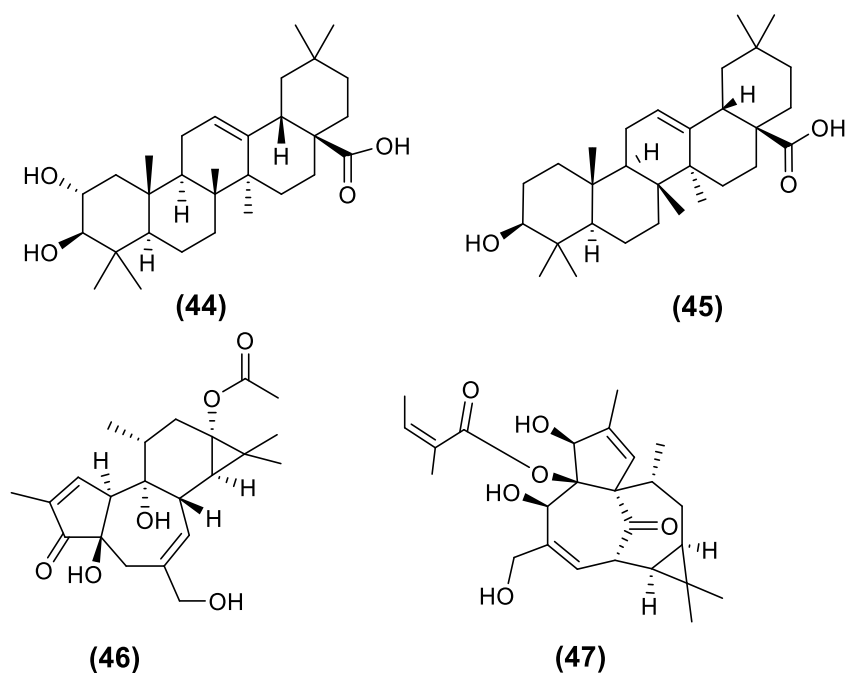


Figure 9: Masilinic acid (**44**), oleanolic acid (**45**), prostratin (**46**), and ingenol mebutate (**47**)

1.4.4 Coumarins as anti-HIV agents

Coumarins are a class of compounds with anti-HIV properties that depend on substitution patterns.^[79] A very good example of such is calanolide A and calanolide B. Calanolide A is

currently used as an experimental non-nucleoside reverse transcriptase inhibitor while calanolide B has less activity and is still undergoing clinical trials.^[36,19]

1.4.5 Phenolic compounds anti-HIV agents

Phenolic compounds are characterized as aromatic secondary metabolites that have species and their distributed across a wide range of plant species and in most edible plants (Figure 10).^[24] The consumption of phenolic rich plants is encouraged to promote a healthy lifestyle and the prevention of diseases.^[80] Phenolic compounds have been extensively investigated for their pharmacological benefits.^[77]

Most phenolic compounds, especially flavonoids have displayed antiretroviral properties such as anti-HIV by inhibiting the reverse transcriptase enzyme and viral replication.^[81] Flavonoids modulate several stages in the replication cycle of HIV and they have the ability to act as entry inhibitors.^[36,82] Compounds such as geranin A (**48**) have been isolated from the plant *Phyllanthus amarus*, this compound has demonstrated good anti-HIV properties when used as a binding inhibitor.^[83] It blocks viral entry and inhibits reverse transcriptase, thus, stopping HIV replication.^[83] Epigallocatechin (**49**) isolated from green tea leaf *Camellia sinensis* has demonstrated good antiretroviral activity by inhibiting the enzyme reverse transcriptase and protease kinetics as well as post adsorption entry. Epigallocatechin has been shown to bind with a high affinity on CD4⁺ T-cells and prevents the binding of glycoproteins into the CD4⁺ cells.^[36] Wikstrol B (**50**) isolated from *Wikstroemia indica* is also one of the vast flavonoids that exhibit antiretroviral properties in *in vitro* studies.^[36] Prolonged consumption of polyphenolic rich fruits such as grapes and pomegranate compounds is advocated in HIV positive people. As they restore disturbances in T-cell homeostasis by enhancement of phytohaemagglutinin (lectin)-induced lymphocyte proliferation.^[84]

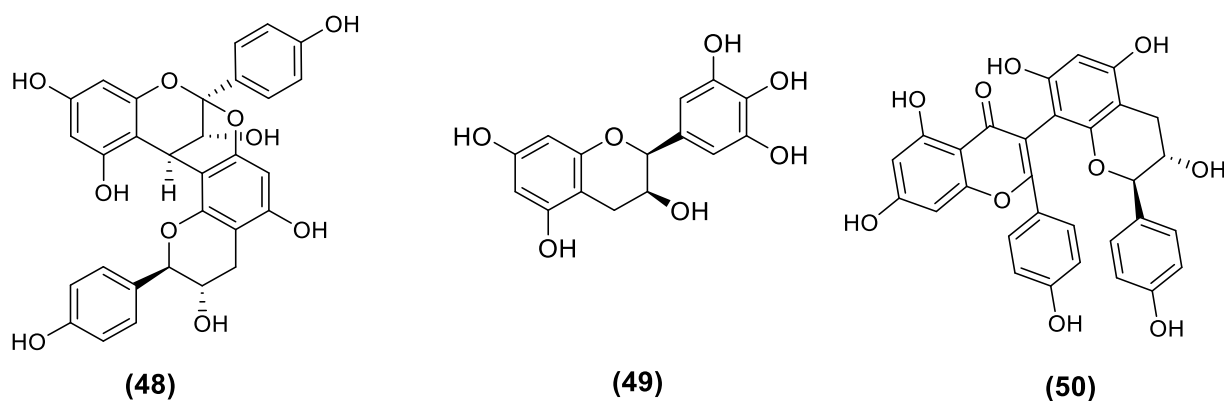


Figure 10: Structures of geranin A (48), wikstrol B (49), and epigallocatechin (50)

There is sufficient evidence that plant based natural product compounds need to be further explored for their anti-HIV properties.^[54] However, it is not all of these compounds that have actually made it to the market or clinical trials but their analogues or synthesised forms.^[54] In addition natural product compounds pose a very difficult challenge as they are found in plants in complex mixtures making them difficult to isolate.^[37]

Most compounds fail to even reach the targeted pharmacological importance as they are metabolised into the body before they could even get to the infected cells.^[54,37] Some natural products need to be consumed in large quantities in order for them to work.^[34,37] This is a problem as it can give rise to cytotoxicity problems making the drug unsuitable for use and defeating the purpose of being an antiretroviral drug.^[70,72,78] There is a need for naturally based pharmacotherapy for viral infection with a good selectivity and direct as natural products offer interesting.^[68,85]

1.5 South African medicinal plants with anti-HIV properties

Many South African plants have been investigated for their anti-HIV properties, following their traditional use by many locals for the treatment of HIV and other sexually transmitted diseases.^[21,76] The HIV inhibitory activity of the South African plants was investigated based on literature review or screening numerous plants against HIV.^[36] Plant extracts using water and organic solvents are also reported to form part of these studies.^[36] Extracts and plants

with good inhibition properties against HIV were further investigated.^[36] Pure and identified active compounds were isolated and investigated for their anti-HIV properties against the essential enzymes of HIV such as reverse transcriptase, integrase and protease.^[37] These were also reported to be investigated for their potential as entry inhibitors.^[64]

Many organic extracts from different plant species and plant parts of South Africa have been shown to exhibit good anti-HIV properties.^[34] Some studies revealed the methanol extract of *Peltophorum africanum* to exhibit good anti-HIV properties against two HIV chemokine receptors CCR5 and CXCR4 tropic viruses.^[22] The study led to isolation of an anti-HIV compound betulinic acid with good anti-HIV properties with high selective indexes.^[37] Additionally, gallotannin isolated from *P. africanum* has been shown to exhibit both polymerase and RNase inhibition properties with IC₅₀ of 6.0 µM and 5 µM respectively.^[37] Catechin (**51**) was revealed to have good inhibition properties in the enzyme integrase (Figure 11).^[87]

The anti-HIV properties of *Cassine transvaalensis* a South African medicinal plant reported for its traditional use in the treatment of HIV were reported.^[37,77] It was shown that the compounds and extracts of this plant had a selective index of 57.1 and an EC₅₀ of 3.5 µg/ml. Some compounds isolated from *Elaeodendron transvaalense* were observed to have a potential to inhibit HIV-1 reverse transcriptase.^[37,77] These compounds lup-20(30)-ene-3,29-diol, lup-20(29)-ene-30-hydroxy-3-one and 4'-O-methylepigallocatechin were investigated for their potential as enzyme reverse transcriptase inhibitors. The results showed good inhibition activities in the extract however was not good for the compounds.^[37,77]

Hypoxis hemerocallidea is one of the most famous South African medicinal plants in the treatment of HIV amongst traditional health practitioners.^[12,26,88] The plant possess many medicinal properties ranging from anti-bacterial and anti-fungal properties present in the plant.^[26] The plant has been widely investigated for its anti-HIV properties following its wide use as a treatment for HIV. Phytochemical studies on the plant extracts revealed the presence of phenolic and flavonoids.^[8,12,22,24,26,88]

Boerhavia erecta is reported to be traditionally used by people of South Africa for the treatment of sexually transmitted diseases and HIV.^[37,77] The plant is reported to have the potential of inhibiting the enzyme integrase and the responsible compound is reported to be rutin (**52**) (Figure 11). *Boerhavia coccinea* is also reported for its traditional use in HIV, diarrhoea and other infectious diseases.^[42] The reverse transcriptase inhibitory properties in the plant were due to the presence of β -sitosterol (**53**) and friedelin (**54**) (Figure 11).^[37,77]

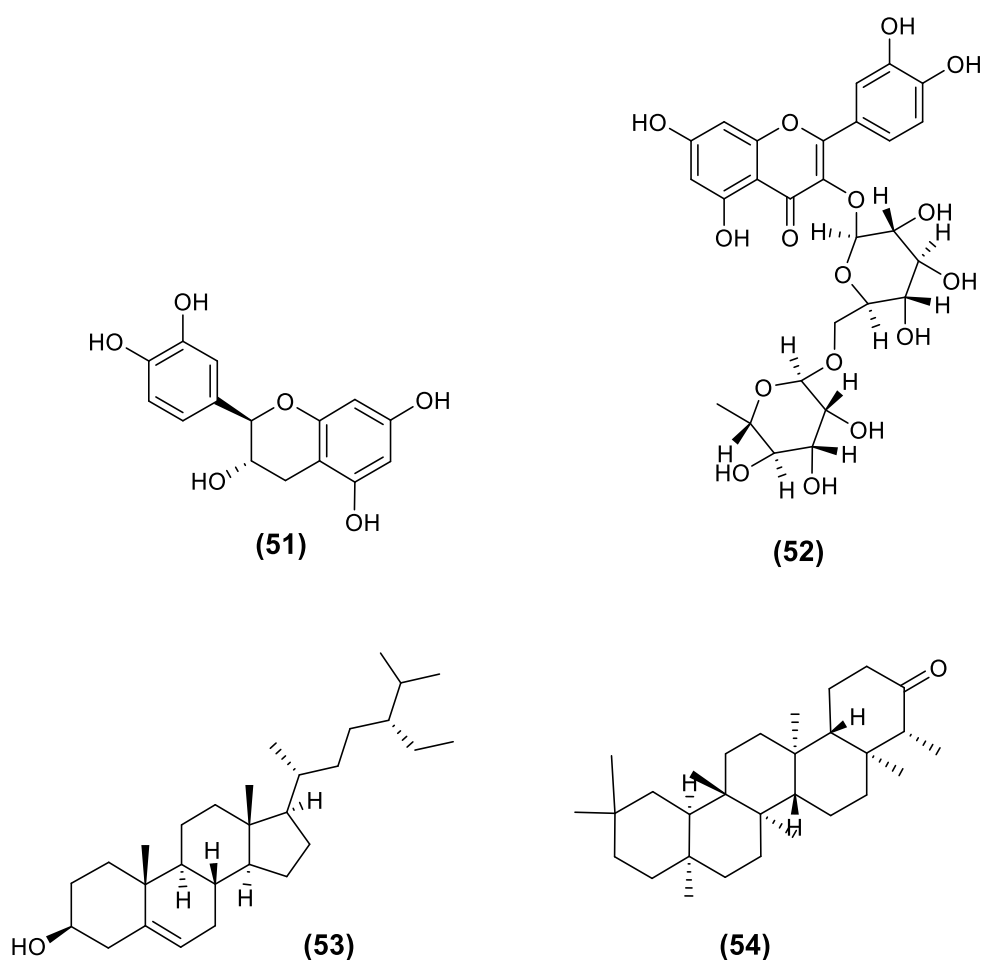


Figure 11: Structure of catechin (**51**), rutin (**52**), β -sitosterol (**53**), and friedelin (**54**)

The potential of South African plants as anti-retroviral agent has been reported in numerous reports.^[21,34] The reports showed the presence of compounds that have different binding sites in HIV enzymes.^[62,76] Nonetheless, there are still many plants that need to be investigated for their potential as anti-retroviral agents.^[37]

1.6 Research problem

The use of conventional therapeutic regimes for the treatment of HIV has shown a great deal of success with enormous down sides as well.^[47,51,67] This is shown by the decrease in HIV related mortality as well as a decline of patients who progress to AIDS.^[33] The challenges are due to the fact that the available therapies do not cure the disease but suppress the virus to undetectable limits.^[47,51,66,69] Other limitations include rapid emergence of viral resistance to drugs, drug toxicity, limited availability and high cost of current anti-retroviral drugs.^[82] Mainstream treatment regimens have often excluded medicinal plants.^[19,71,75] This is despite studies showing that plants can be a source of new drug leads with different structural compounds.^[18]

Many pharmaceutical companies have stopped investigating anti-HIV properties of plants.^[4] Compounds in plant mixtures are very complex as such there are challenges such as taking too long to isolate one pure single compound, solubility problems, and the loss of activity in the compound isolated from a crude extract with good anti-HIV activity.^[52,19] However, the decline in new chemical entities in drug development has re-ignited the interest in natural products as they have proven to be a reliable source of new drug leads.^[4,5,89]

Research conducted in South Africa have reported claims made by traditional health practitioners concerning plants that can cure AIDS and other sexually transmitted diseases.^[18,24,31,37,90] However, most of these plant mixtures have not been investigated for their HIV potency, lead compounds isolation as well as their mode of action.^[19,48,53,90] According to the World Health Organization (WHO), a traditional medicine needs to be scientifically validated for safety, efficacy and administration before it reaches the market with a known active ingredient.^[89] This study will provide sufficient evidence by identifying the active ingredient, efficacy and safety of four medicinal plants used by a local traditional health practitioner with claims of treating and eradicating HIV by using the mixture of the four plants. The study will also provide data on the potential of the medicinal plants and the herbal mixture to completely

eradicate HIV in the body of infected individual by activation of latent cells infected by the virus.

1.7 Aim of the study

The research aims to evaluate the potential of a locally used traditional mixture in the treatment of HIV by providing scientific evidence with proven efficacy for the traditional use of the medicine as an anti-viral medicine as well as its potential to eradicate HIV latency, identify the active ingredient for the standardization of the herbal mixture, and lastly produce a standardized extract for further development.

1.8 Research objectives

The specific objectives of the study were:

- Collection and identification of the four different plant species obtained from the traditional health practitioner.
- Extraction of the four plants using different solvents of varying polarities.
- Chemical fingerprinting of active extracts, fractions and compounds using UPLC-QTOF-MS.
- Fractionation of active extracts using column chromatography, flash chromatography, HPLC and liquid-liquid partitioning.
- Determination of the anti-HIV activity of extracts, fractions and compounds using a DeCiPhR assay.
- Determination of the latency reversal activity of extracts, fractions and compounds
- Isolation of compounds using chromatographic techniques such as LC-SPE-UV-MS-NMR and HPLC-MS

- Elucidation of chemical structure of pure active compound using 1D, 2D NMR and mass spectrometry
- Semi-pilot scale development of a standardized traditional medicine from *G. sericocephala* and the four-plant mixture using pharmaceutical accepted solvents for use in the treatment of HIV and HIV latency reversal.
- Quantification of active compounds in the developed product to be used for batch-to-batch production quality control purposes.

1.9 Outline of the study

Chapter one

Chapter one is focuses on the general introduction of medicinal plants, introduction of HIV and replication cycle and the use of medicinal plants in the control of HIV infection.

Chapter two

Chapter two focuses on the different extraction methods used in the study including the use of pharmaceutically accepted solvents on laboratory scale extraction for the development of product Nkabinde. The chapter also discusses the anti-HIV and HIV latency reversal activity of all obtained extracts as well as the chemical characterization of the most active extracts including the four plants present in the four-plant mixture separately obtained from the traditional health practitioner using UPLC-MS.

Chapter three

Chapter three focuses on different fractionation techniques used for the isolation of the active compounds and the chemical biomarkers in the DCM extract of *G. sericocephala*. The chemical analysis of all fractions using UPLC-QTOF-MS-MS and the elucidation of chemical structures of all isolated compounds in the study using 1D and 2D NMR spectroscopy is also

discussed. The HIV replication inhibition of the compounds, the HIV latency reversal activity and cytotoxicity of the active compounds isolated in *Gnidia sericocephala* is also discussed.

Chapter four

This chapter focuses on the extraction optimization for the development of product Nkabinde using pharmaceutically accepted solvents in a GMP (Good Manufacturing Practises) compliant environment (a semi-pilot scale extraction). The chapter also focuses on the anti-HIV and latency reversal activity of the developed products as well as their chemical characterization using UPLC-MS. Lastly, the study also focuses on the quantification of the most anti-HIV active compound in the developed extracts.

Chapter five

Chapter five focuses on the overall conclusion of the study.

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Chapter two

The anti-HIV activity and chemical profiling of the four plants used for treatment of HIV labelled as product Nkabinde

2.1 Background

In 2000, the World Health Organisation (WHO) made recommendations for countries to have locally produced traditional medicines and their integration into the primary health care sector, from which South Africa is not exempted.^[1] The country with its rich biodiversity has implemented these recommendations, whereby, a traditional health practitioner needs to provide scientific evidence to support the claims of a particular traditional medicine.^[2] The scientific data needed for the medicine to be commercialised and approved for human consumption include the effectiveness of the traditional medicine, its safety, dosages, active ingredient specific for a certain disease, its stability, as well as quality control of the traditional medicine for batch to batch production.^[2] This study is also trying to implement the recommendations as regulated by WHO.^[3] A case study is *Sutherlandia frutescens* an indigenous medicinal plant of South Africa, has been widely reported for its range of activities, which includes its anti-HIV activity.^[3] The plant has undergone clinical trials to validate its safety and efficacy using human participants and was shown to delay the progression of HIV to AIDS.^[4] A study by Wilson *et al.*, (2015) revealed that *S. frutescens* powder does not affect viral load in patients with a CD4 T-lymphocytes with a viral load of more than 350 cells/ μ l at a dose of 80 mg/kg over three months.^[5] The South African Medical Research Council has also reported the safety of the plants leaf powder after being given to vervet monkeys.^[6] The plant powder showed no sign of toxicity even after being given a dose equivalent for human consumption in a period of 9 months^[6]. The HIV efficacy of this plants was reported to be due to the presence of L-canavanine. When tested *in vitro* the compound showed efficacy of 95% in infected lymphocytes.^[4]

A South African traditional health practitioner (THP) Nkabinde reports to be using traditional medicine for the treatment of HIV and other various diseases such as sexually transmitted

diseases and other opportunistic diseases (The documented information provided to the University of Pretoria). THP Nkabinde uses a mixture of four different plants for the treatment of HIV and other opportunistic diseases, *Sclerocarya birrea* bark, *Gnidia sericocephala* roots, *Senna italica* roots and *Pentanisia prunelloides* roots. Nkabinde also uses the mixture of these four plants to boost the immune system in HIV infected patients and to treat other STDs such as gonorrhoea, kaposi sarcoma as well as an appetite booster. The stem bark of *S. birrea* is combined with the roots of *S. italica*, *P. prunelloides* and *G. sericocephala*, dried, ground and then boiled in water for 5-8 min. After cooling the solid material is separated, and the decoction (water mixture) is used. The decoction is taken three times a day (4 tablespoons) depending on the severity of the disease the dosage can be taken up to 250 mL as a single dose. Nkabinde reported that his treatment does not have side effects and there is no known toxicity for his treatment. The full scientific evidence to support his claims is however lacking.

To substantiate the claims made by THP Nkabinde, the efficacy and safety of the plants was determined in this study. This chapter focuses on the preparation of different extracts from four different plants, biological screening against HIV using a dual enhancement of cell infection to phenotypic resistance assay and the reversal of HIV latency. The chemical characterization of the biologically active extracts was determined using UPLC-MS to determine their chemical fingerprint.

2.2 *Senna italica*

2.2.1 Description and ecological distribution

Senna italica a plant belonging to the *Fabaceae* family, is a herbaceous perennial medicinal plant that grows up to a height of 60 cm high.^[5] The leaves of *S. italica* are alternate and smooth, with erect smooth stem. Its flowers are pale-yellow, and legumes are oblong, with membranous smooth elevated ridges upon the valves.^[6] The plant flowers during summer and autumn. *S. italica* is widely distributed in tropical Africa, Madagascar, America and

Australia.^[7,8] In South Africa *S. italica* is regarded as a native plant, and is mainly distributed in the southern parts of the country.^[8]

2.2.2 Traditional uses of *Senna italica*

S. italica leaves and pods are traditionally taken as infusions and used as a purgative. Its crushed seeds are used as a decoction and as a laxative.^[7] and hence its usefulness as either for habitual constipation or for occasional use.^[6] Other *Senna* species are widely used to treat ulcers, calculi in the urinary tract infections and other sexually transmitted diseases.^[7] In South Africa, the vhaVenda people use the water extracts of some *Senna* species to treat sexually transmitted infections and as anti-bacterial agents.^[5]

2.2.3 Reported anti-HIV properties of *Senna italica*

The anti-HIV properties of *S. italica* extracts have been reported.^[9] The ethanol extract of *Senna italica* was investigated for its anti-HIV activity against recombinant HIV-1 enzyme using non-radioactive HIV-RT colorimetric assay. ^[9] The percentage inhibition of *S. italica* against enzyme transcriptase was reported to be greater than 30% at a concentration of 0.1 mg/ml.^[9] Compounds such as emodin and chrysophanol which have been isolated from some *Senna* species have been found to exhibit anti-HIV activity against integrase.^[10]

2.2.4 Phytochemical analysis of *Senna italica*

A variety of compounds have been isolated and identified from different plant parts of *S. italica*.^[10] These compounds include anthraquinone and its derivatives such as emodin (**55**), aloin (**56**), physcion (**57**) and chrysophanol (**58**) from the roots of the plant.^[10] Anthraquinones are a class of compounds reported to exhibit laxative properties, antimalarial and antineoplastic properties.^[10] Emodin has been identified in a traditional Chinese formulation to exhibit antiviral properties against severe acute respiratory syndrome coronavirus 2 (SARS-

COV-2).^[11] Emodin has also been reported to have good anti-bacterial, anti-inflammatory as well as laxative properties.^[10] The flavonoid compound rutin (**59**) exhibiting quite a number of pharmacological properties has also been isolated from *S. italica*. Rutin has been reported to have a wide range of pharmacological activities such as antibacterial, antiprotozoal, antitumor, anti-inflammatory, anti-allergic, antiviral, cytoprotective, vasoactive, antiplatelet, antispasmodic, and antihypertensive properties.^[10,12,13] This evidence supports the traditional use of *Senna italica* by local people as a laxative and its use in the treatment of sexually transmitted infections. Triterpenoids have also been isolated from *S. italica*, these include β -sitosterol (**60**) and daucosterol (**61**).^[5,6] Triterpenoids are well known for their good anti-cancer properties.^[14] This can be used to support the traditional use of the plant as treatment for ulcers.^[8] The structure of compounds isolated from *S. italica* is shown in Figure 2.1.

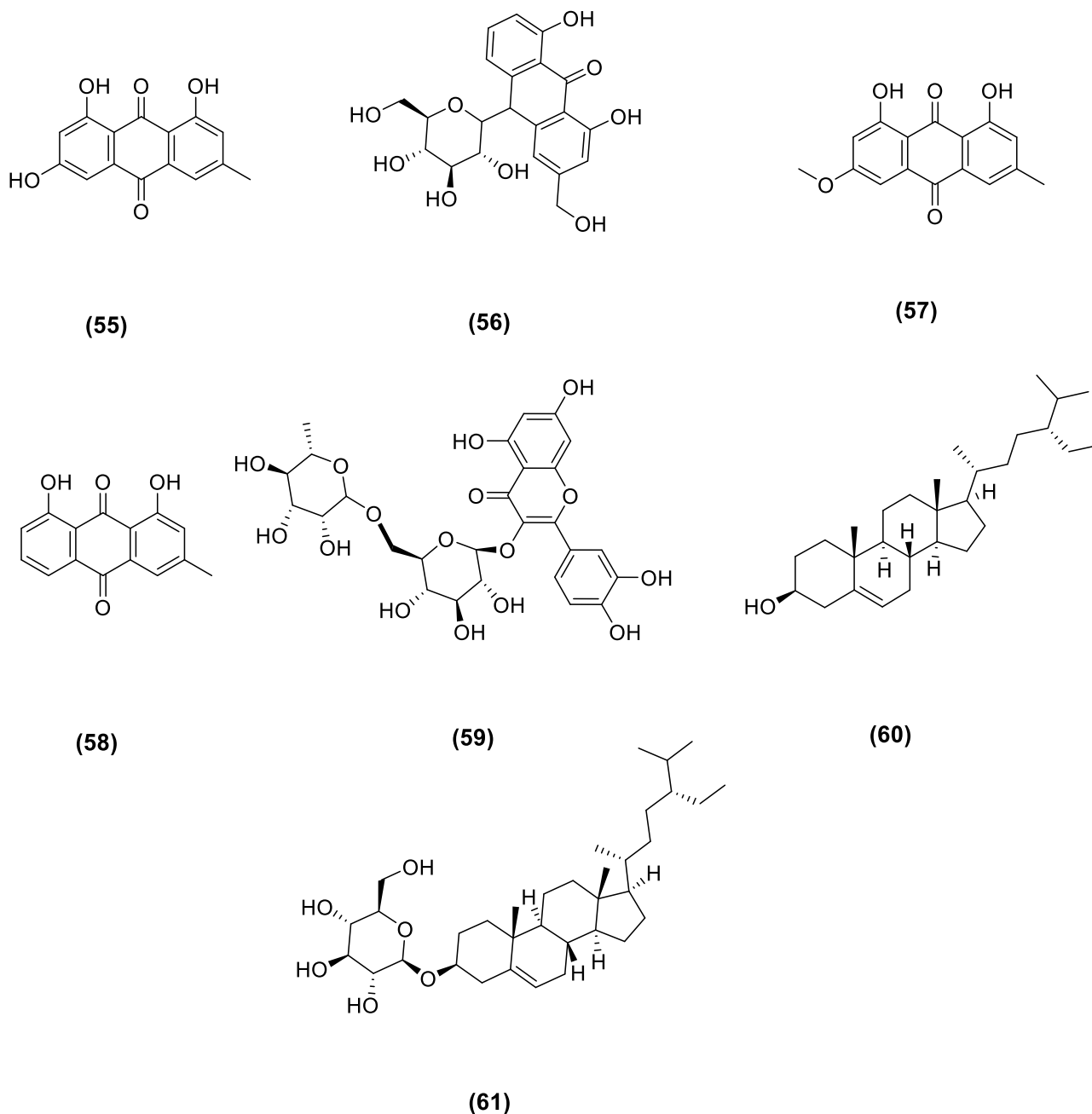


Figure 2. 1: Emodin (55), aloin (56), physcion (57) and chrysophanol (58), rutin (59), β -sitosterol (60), and daucosterol (61)

2.3 *Sclerocarya birrea*

2.3.1 Description and ecological distribution of *Sclerocarya birrea*

Sclerocarya birrea belongs to the *Anacardiaceae* family and it's a drought resistant plant.^[15]

The plant is commonly known as marula or the tree of life due to its bearing fruit properties.^[16]

The marula fruits have a thick yellow peel with translucent white flesh.^[17] *S. birrea* is mainly

distributed in the southern parts of Africa.^[18] In South Africa the marula tree is mainly found in Gauteng, KwaZulu Natal, Eastern Cape, Limpopo and Mpumalanga provinces.^[19]

2.3.2 Traditional uses of *Sclerocarya birrea*

S. birrea plant has been reported for its wide range of traditional uses.^[20] The marula fruit is mainly eaten fresh and is also processed into beverages, jam and jellies.^[18] The fruit kernels of marula are also edible as they are usually dried and eaten to supplement and preserve food especially leafy vegetables.^[18] The fatty oil found in marula kernels is used as a food flavouring and to soften food.^[21] In general, marula fruit has a sweet and sour taste juice because of its high contents of vitamin C when compared to citrus juices.^[22] In South Africa, the decoction from marula stem bark is traditionally used for the management of pain and inflammation.^[21] It is also used to treat ailments such as stomach-ache, insect bites, epilepsy, rheumatism.^[21] Other communities administer the decoction orally and anally to induce vomiting and diarrhoea respectively.^[20] Some African countries use the bark of the plant to treat bacterial infections.^[23] The people of Sudan use dried bark of marula in wound healing and boiled infusions used for the treatment of malaria.^[24] The wide use of marula in African countries led to the commercialization of the plant as balms for treating nose, ear and throat conditions.^[25]

2.3.3 Anti-HIV properties of *Sclerocarya birrea*

The use of *S. birrea* extracts in the treatment of HIV mainly focuses on the opportunistic infections that arise due to HIV and AIDS.^[18,26] These infections include oral candidiasis which is more common in HIV positive individuals.^[18] Masoko *et al.*, (2008) reported the antifungal properties from the methanol extract of *S. birrea* stem bark against a variety of *Candida* species such as *Candida (albicans, glabrata, parapsilosis, tropicalis, and krusei)* by using a broth micro dilution method.^[18] The results showed *S. birrea* to inhibit growth against different *Candida* strains with an MIC of 0.5 mg/ml.^[22,23] The extract was also reported to inhibit

Cryptococcus neoformans, a fungus that produce pneumonia and meningitis in HIV positive individuals.^[22] Compounds such as gallic acid, epigallocatechin have been isolated from *S. birrea* and exhibit anti-HIV activities by inhibition of the enzymes integrase and reverse transcriptase.

2.3.4 Phytochemical analysis of *Sclerocarya birrea*

S. birrea has been reported to be a rich source of anti-bacterial and anti-oxidative compounds.^[23,26] Russo *et al.*, (2013) reported the presence of catechin (62), catechin derivatives and high levels of tannins such as procyanidin oligomers which have been revealed to possess anti-oxidative properties.^[27] Flavonoid glycosides such as quercetin-3-O- β -D-(6'-galloyl)-glucopyranoside (63), quercetin-3-O-arabinoside (64), gallic acid (65) have also been reported in *S. birrea*.^[27] The stem bark of *S. birrea* was shown to constitute a variety of compounds ranging from flavonoids, tannins, alkaloids, saponins, fatty acids and sterols. Quercetin (66), epigallocatechin gallate (67) and procyanidins such as procyanidin B2 (68) have been identified and isolated from *S. birrea*.^[28–30] The structure of compounds isolated from *S. birrea* is shown in Figure 2.2.

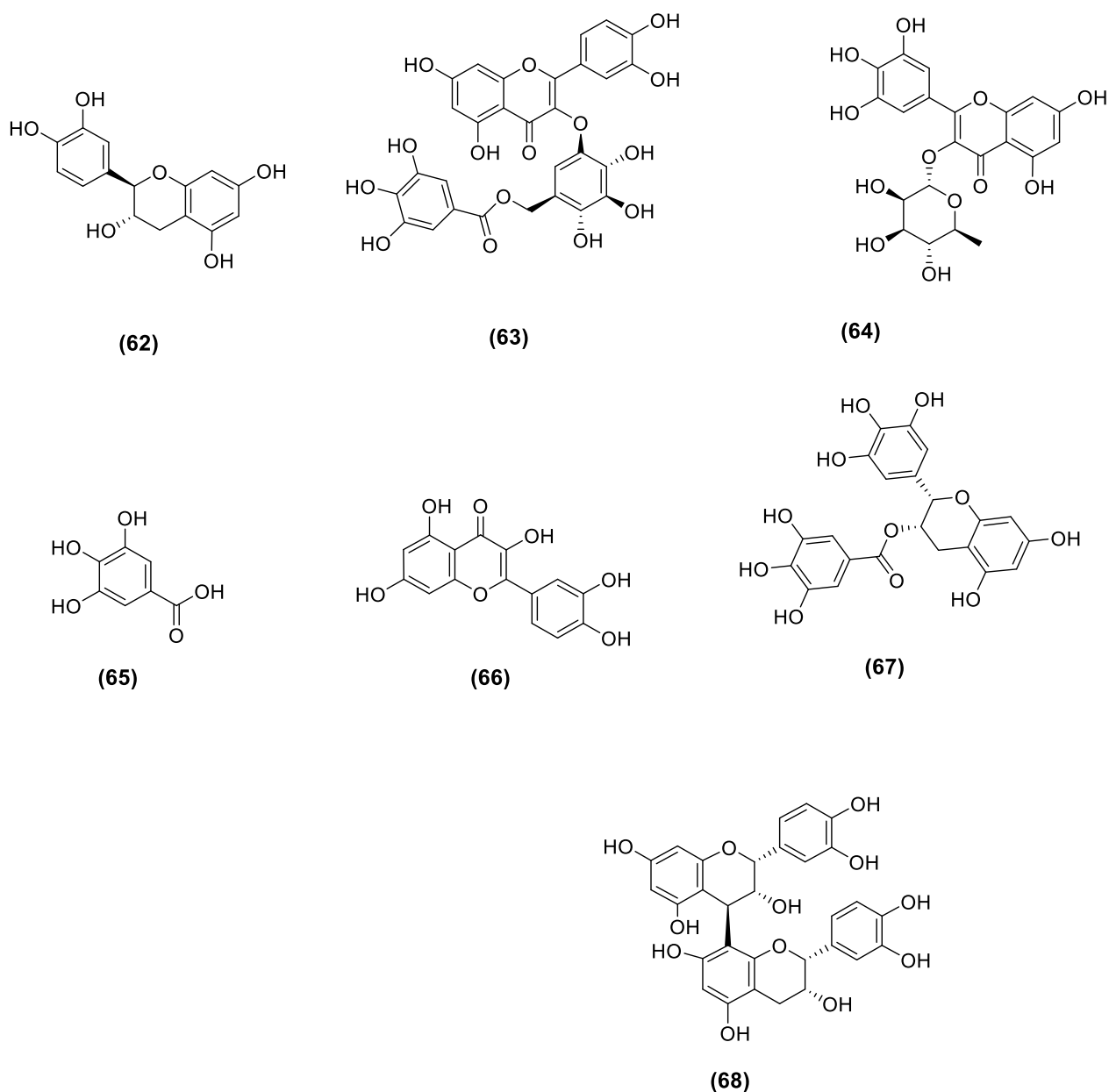


Figure 2. 2: Catechin **(62)**, quercetin-3-*O*- β -*D*-(6'-galloyl)-glucopyranoside **(63)**, quercetin-3-*O*-arabinoside **(64)**, gallic acid **(65)**, quercetin **(66)**, epigallocatechin gallate **(67)**, and procyanidin B2 **(68)**

2.4 *Pentanisia prunelloides*

2.4.1 Description and ecological distribution of *Pentanisia prunelloides*

Pentanisia prunelloides is a medicinal plant belonging to the *Rubiaceae* family. It is an erect perennial herb with stout hairy stems that grows approximately up to a height of 30-60 cm from the rootstock. ^[31] It has broadly ovate to oblong hairy leaves with tubular purple-blue

flowers that occur in spring.^[31] In South Africa, it is widely distributed in the grasslands of Gauteng, Mpumalanga, Kwa-Zulu Natal, Free State and some parts of Eastern Cape province.^[31]

2.4.2 Traditional use of *Pentanisia prunelloides*

P. prunelloides is widely used because of its curative properties found mainly in its tuberous roots and sometimes leaves.^[32] *P. prunelloides* is traditionally used in the treatment of inflammation, infectious viruses, muscular pains, haemorrhoids, tuberculosis and snake bites.^[33,34] This plant is also used to treat palpitations, urinary tract infections, sexually transmitted infections such as genital warts/herpes, chlamydia, gonorrhoea and perforated peptic ulcers.^[31,32,34] In the early nineteenth century, the decoction from the roots were used as a cure for the influenza epidemic.^[32–34] The infusion of the roots is used as a purgative for stomach cramps. The plant concoction is also internally and externally used for the treatment of piles and tuberculosis. The local people of KwaZulu Natal use infusions of *P. prunelloides* to ease pains during labour. Consequently, the infusions are also taken to treat infertility, as the plant is believed to prevent muscular contractions of the uterus.^[31,32]

2.4.3 Anti-HIV properties of *Pentanisia prunelloides*

The aqueous extract of *P. prunelloides* have been reported to possess anti-viral properties.^[32] *P. prunelloides* constitutes of (-)-epicatechin as one of its major constituent.^[35] This compound has been isolated before from green tea and screened against HIV. It was found that (-)-epicatechin possess reverse transcriptase inhibition activities. Luteolin a major component of *Morinda citrifolia* which belongs to the family *Rubiaceae* has also been detected in *P. prunelloides* and has been found to possess anti-HIV properties.^[36] The compound was shown to be effective against the HIV enzyme protease and was found to interfere with HIV long terminal repeats.^[36]

2.4.4 Phytochemical analysis of *Pentanisia prunelloides*

The phytochemistry of *P. prunelloides* has been investigated due to the pharmacological properties of the plant and its wide traditional uses against different ailments.^[31] The plant was revealed to constitute of saponins, flavonoids, tannins, anthocyanidins, anthraquinones, triterpenoids and sugars. Some phytochemical investigations on the plant led to the isolation of compounds such as oleanolic acid (**69**), diosgenin (**70**), (-)-epicatechin (**71**).^[32,33] The structure of compounds isolated from *P. prunelloides* is shown in Figure 2.3.

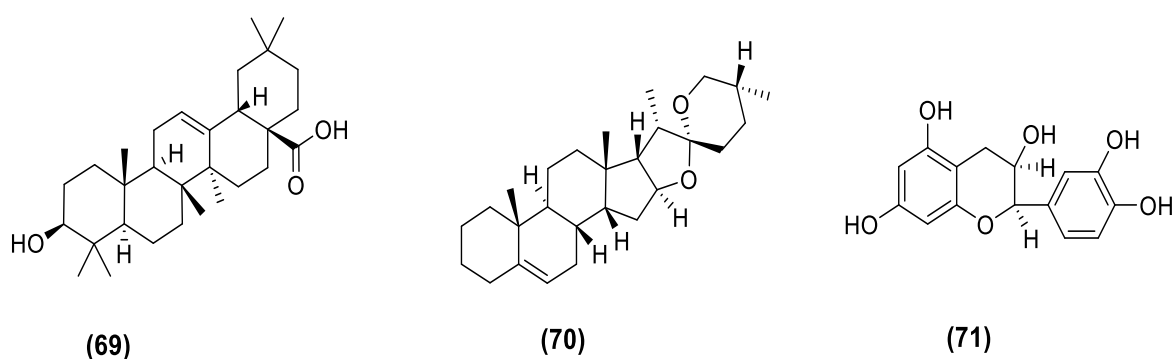


Figure 2. 3: Oleanolic acid (**69**), diosgenin (**70**), and (-)-epicatechin (**71**)

2.5 *Gnidia sericocephala*

2.5.1 Description and ecological distribution of *Gnidia sericocephala*

Gnidia sericocephala belongs to family Thymelaeaceae.^[37] This species is reported to have pentamerous flowers.^[38] It is reported to be widely distributed in Southern Africa, in South Africa the species can be found in the Free State, KwaZulu Natal, Gauteng and Mpumalanga Province.^[39] The ecological distribution of the plant is not well documented as there is little information on it.

2.5.2 Traditional uses of *Gnidia sericocephala*

Species of this *genus* have been used throughout South Africa as traditional treatment for various ailments such as conception, childbirth, burns, asthma, malaria, insanity, pulmonary

tuberculosis and poor appetite.^[38,39] The Basotho people inhale the smoke of *Gnidia anthylloides* species to treat fever and to stabilise heart conditions.^[39] Other species from this genus have been reported to be used for the treatment of botulism and anthrax in cattle.^[39] *Gnidia* species are also known for their severe irritant effects that causes blistering.^[40] The people of Madagascar make use of the strong fibrous bark from some species of *Gnidia* to make ceremonial clothing, paper and twine.^[39] These traits are reported to be due to the presence of toxic coumarins and diterpenoids they contain.^[37,39] Cases of deaths in humans and animals have also been reported due to *Gnidia* poisoning.^[39]

2.5.3 Anti-HIV properties of *Gnidia sericocephala*

The genus *Gnidia* is one of many genera from Thymelaeaceae family.^[14] Species from other genus having extracts with reported anti-HIV activities.^[41] These extracts include a dichloromethane extract of *Daphne gnidium* aerial parts.^[42] The extracts showed 50% anti-HIV activity in a cell based assay with HIV full life cycle.^[42] The extract also showed the same anti-HIV properties in different viral isolates that only target the entry and binding site of the HIV, with an IC₅₀ of approximately 280 ng/ml. Plants from the genus *Gnidia* have been reported to contain compounds that are responsible for combating HIV known as daphnane diterpenes.^[37,43] Daphnane diterpenoids include compounds such as gnidimacrin (**72**), gnidilatidin (**73**), gnidicin (**74**), winstroelide A (**75**) pimelea factor P2 (**76**) amongst others. A review by Yue-Xian et al (2019) confirmed the strongest anti-HIV properties of daphnane diterpenoids to be from those with orthoester moiety at C-9, C-13 and C-14 when compared to other rearrangements especially those with lactone ring such as gnidimacrin.^[44] Gnidimacrin has the ability to inhibit HIV-1 at picomolar concentrations and was also act as a good candidate in the activation of latent HIV by killing the persistently infected HIV-1 cells and inhibit R5 viruses at picomolar levels.^[45] The structure of compounds identified from *G. sericocephala* with reported anti-HIV activity is shown in Figure 2.4

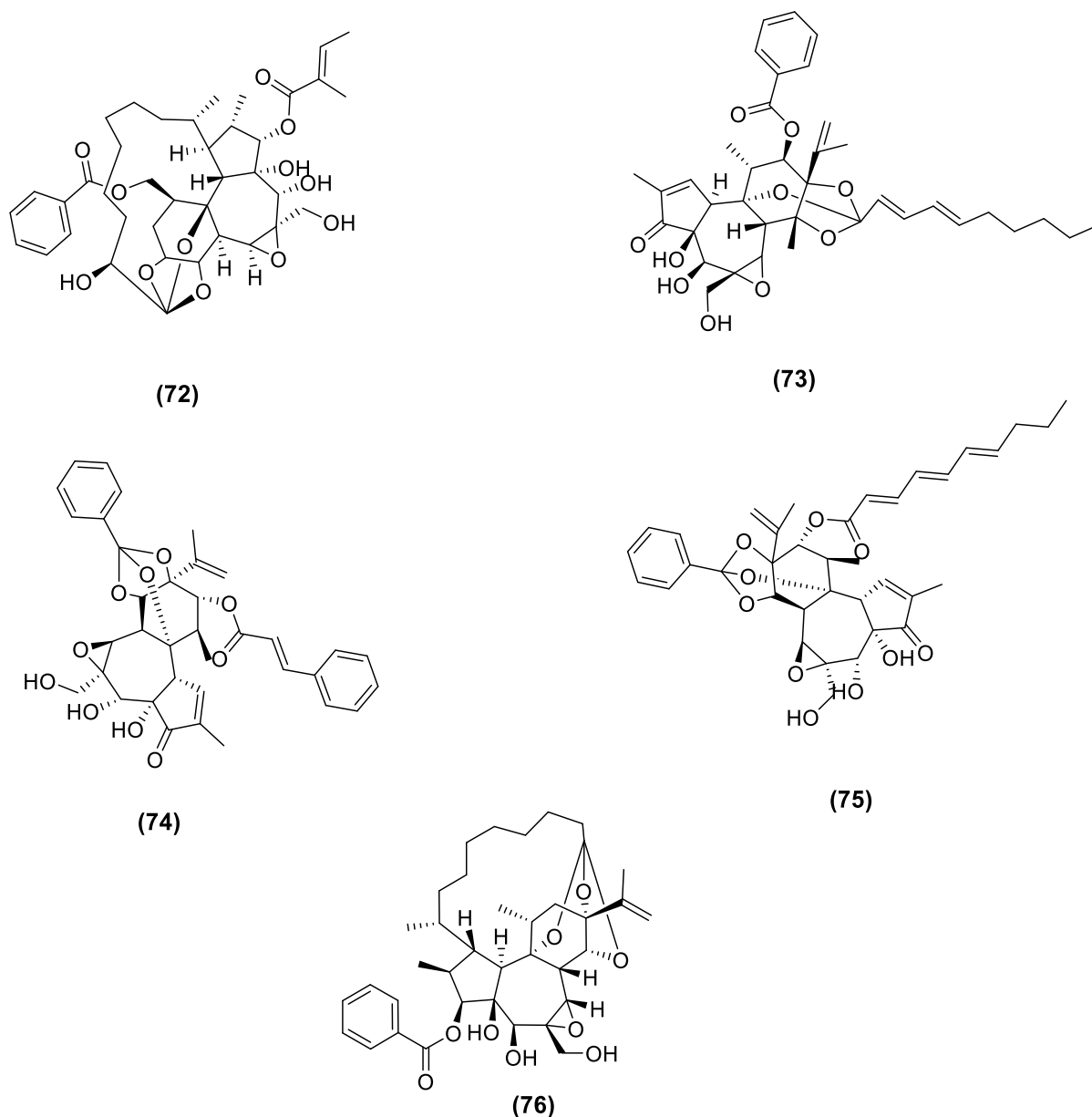


Figure 2. 4: Gnidimacrin (**72**), gnidilatidin (**73**), gnidicin (**74**), winstroelide A (**75**) pimelea factor P2 (**76**)

2.5.4 Phytochemical analysis of *Gnidia sericocephala*

Phytochemical analysis on *Gnidia* species have shown the plants to constitute of a variety of compounds belonging in different classes such as, coumarins, benzophenone glycosides, phorbol esters, daphnanes diterpenes, flavonoids, chromones, lignans, as well as neolignans. Phytochemical studies on *Gnidia socotrana* leaves and twigs led to the isolation of 6-(8"-umbelliferyl)-apigenin (**77**) and 7-7'-dihydroxy-3-8'-biscoumarin (**78**).^[37] 2,4',6-trihydroxy-4-

methoxybenzophenone-2-O-glucoside **(79)** and 2,3,4',5,6-pentahydroxybenzophenone-4-C-glucoside **(80)** are benzophenone glycosides isolated from the aerial parts of *Gnidia involucre*.^[46] Prostratin **(81)** a phorbol ester with anti-HIV and HIV latency reversal properties has been reported and isolated from *Gnidia involucre*.^[47] The phytochemistry of *Gnidia* species can be used to support its medicinal use by local people. The structure of compounds identified and isolated from *G. sericocephala* is shown in Figure 2.5.

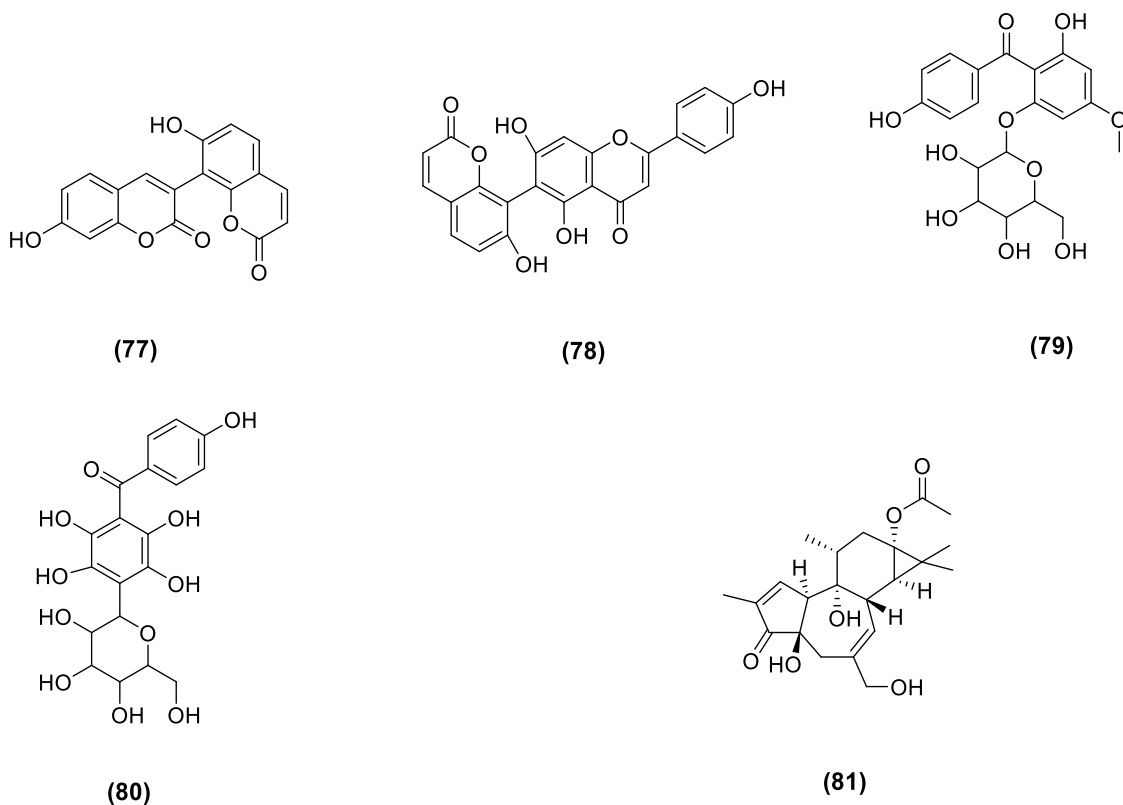


Figure 2. 5: 6-(8''-umbelliferyl)-apigenin **(77)**, 7-7'-dihydroxy-3-8'-biscoumarin **(78)**, 2,4,6-trihydroxy-4-methoxybenzophenone-2-O-glucoside **(79)**, 2,3,4',5,6-pentahydroxybenzophenone-4-C-glucoside **(80)**, and prostratin **(81)**

2.6 *In vitro* biological assays against HIV

The development of optimal anti-retroviral treatment regimens has allowed for the improvement of *in vitro* anti-HIV biological screening in compounds.^[48] These assays allowed for the extensive measurements of infections in the presence of a candidate compound with increasing concentrations. This is either done using a single-round or a multi-round infectivity assay.^[49] Single-round infectivity assay is done by infecting primary CD4⁺ T cells with a

pseudo virus^[48-49]. A pseudo virus has defective *env* viral genome^[49]. The defective viral genome allows for the measurement of compounds with viral entry inhibitory effects and cannot detect inhibitors of viral budding and virion infection as the virus only replicate in a single cycle.^[49] Multi-round infectivity assays make use of a replicating virus. In this assay the virus is cultured with a cell line and is quantified by detecting the viral antigen p24 or reverse transcriptase after several days or weeks in the presence of the candidate compound. The advantages of using multi-round infectivity assay over a single-round infectivity assay is the ability for multi-round infectivity assay to detect late phase inhibitors and the selection of candidate inhibitors with different modes of action.^[50] For this study a multi-round infectivity assay known as the dual enhancement of cell infection to phenotype resistance assay to investigate the anti-HIV activity of the four plants is used.^[42]

2.6.1 Background on dual enhancement of cell infection to phenotype resistance assay

The dual enhancement of cell infection to phenotypic resistance assay (deCIPhR) is a co-cultivation assay that makes use of transfected cells and reporter cell lines.^[51] The transfected cells are directly co-cultivated in the presence of extracts with reporter cell lines. During co-cultivation, there is a direct and rapid cell to cell viral transmission as shown in Figure 2.6. The inhibitory effects of the extract or drugs replication is monitored using beta galactosidase. In the presence of the compounds if there is activity the colour of o-nitrophenyl- β -d-galactopyranoside (ONPG) will change from yellow to white and if there is inactivity the cells will remain yellow.^[51,52]

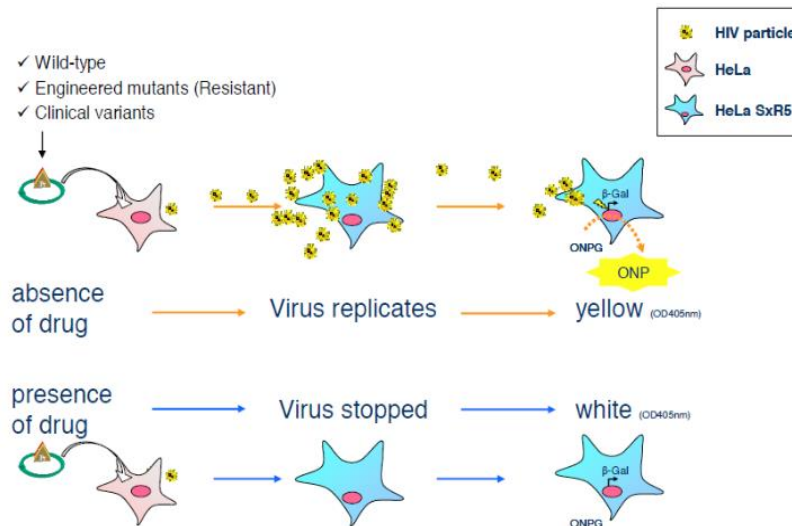


Figure 2. 6: Diagram for the dual enhancement cell infection to phenotype resistance (deCIPhR assay)- provided by Prof.Thomas Klimkait.^[52]

2.6.2 Activation of latent HIV reservoirs

Antiretroviral therapy has allowed for the suppression of HIV replication to undetectable limits.^[53] This has reduced HIV related morbidity, prolonged survival rates and has prevented HIV transmission. However, prolonged use of ART can cause an emergence of HIV drug resistant variants as it is unable to completely eradicate the virus in the body.^[42] This resistance arises due to development of mechanisms that the virus uses to escape the host immune system. All this can be attributed to the fact that anti-retroviral drugs only target actively replicating virions and neglects the dormant virus lying in latent reservoirs.^[54] Latent reservoirs are composed of numerous cell types such as macrophages and HIV infected memory CD4⁺ T cells. Hence, it is essential to completely eradicate the virus in the host by activating memory CD4⁺ T cells, macrophages and all the other cells that acts as reservoirs for the actively replicating virions.^[53,55] Significant therapeutic strategies have been followed to target latent HIV reservoirs, one of these include the "shock and kill" strategy. This method works by activating latently infected CD4⁺ T cells with HIV provirus using latency reversing agents (LRAs). Many LRAs have been identified and investigated and belong to different chemical classes such as the histone deacetylase inhibitors (HDACi) and protein kinase C

(PKC) activators. However, the available LRAs are unable to reduce the size of inducible viral reservoirs in clinical trials, thus, the need for new LRAs with improved efficacy.^[53–55]

2.7 Analytical techniques used in the chemical profiling of plant extracts

2.7.1 Thin layer chromatography (TLC)

Thin layer chromatography also known as TLC is one of the oldest techniques used in the separation of non-polar compounds due to it being fast, least expensive and the simplest chromatographic technique.^[56] This technique makes use of an aluminium foil plate with silica gel embedded on it as the stationary phase. TLC is usually taken as the first step in the purification of compounds as it assists in identifying a good mobile phase prior to column chromatography. Compounds on TLC elute based on their affinity with the stationary and mobile phase. Polar compounds are retained in the stationary phase and move slowly while non-polar compounds elute faster.^[56]

2.7.2 Ultra-performance liquid chromatography coupled with quadrupole-time of-flight mass spectrometry (UPLC-QTOF-MS)

Ultra-performance liquid chromatography (UPLC) is an analytical technique used for the qualitative and quantitative analysis of compounds in complex matrices.^[57] It is used to identify and purify low molecular weight compounds. The technique works on the principle of hydrophobic interactions when using a reverse phased column, where non-polar compounds are retained longer, and polar compounds are eluted first.^[58] The mobile phase used consists of a mixture of water and acetonitrile or water. The column chemistry is mostly dependent on the polarity of targeted compounds. The advantages of using UPLC over HPLC is increased speed, accuracy, resolution, sensitivity, and less flow rates.^[59] Due to the column particle size UPLC systems can handle high pressures with a short run time. For the tentative identification of compounds, a quadrupole-time-of-flight mass spectrometry (Q-TOF-MS) is coupled to the UPLC. The Q-TOF-MS is a hybrid technique with its quadrupole technology working as a mass

filter.^[60] The analyte components are passed through an ionization source known as electrospray ionization source (ESI). ESI produces single charged ions that allow for the analysis in mass analysers with limited m/z range. such as quadrupoles to high resolution mass spectrometers offer a very reliable, sensitive, and significantly reduced analysis time with increased sample throughput and resolution of peaks in crude extracts. This technique allows for the tentative identification of compounds in plant extracts, by providing accurate mass information with low millidaltons (mDa) precision that is less than 5 mDa.^[57] The mass information can be used to predict the elemental composition of the compound and fragmentation ions can be used to confirm identified compounds using literature or libraries such as Chempider and PubChem.^[61,62]

2.7.3 UNIFI Scientific information system

UNIFI software is a Waters Corporation product that uses an automated data processing software platform with scientific libraries of natural product compounds.^[63] The software makes use of the chromatographic and mass spectral components of the extracts from UPLC-QTOF-MS/MS to integrate data acquisition, data mining, and library searching, MS fragment matching, as well as report generation.^[63,64] Its in-built tools such as Chinese Traditional Medicine Library that contains all herbal details found in the 2010 edition of the Chinese Pharmacopoeia, when encompassed together comprises of more than 6000 compounds that are used for confirming compounds in the extracts.^[64] For structural elucidation of unknown compounds, UNIFI software generates an elemental composition calculator that determines a few possible formulas for an accurate mass peak, based on isotope pattern matching along with the fragmentation ion peaks. The chemical composition of compounds given with iFit values greater than 0.8 are considered accurate for confirmation.^[63,64]

2.8 Methodology

2.8.1 Collection and identification of plant samples

S. birrea stems, *G. sericocephala* roots, *S. italica* roots and *P. prunelloides* roots were collected from Tugela Ferry in Msinga region of KwaZulu Natal and delivered by THP Nkabinde, who disclosed the traditional use of the plants to the University of Pretoria. The plants were identified at the H.G.W.J Schweickerdt Plant Herbarium at the University of Pretoria where the voucher specimens were deposited as shown in Table 2.1. The leaf extract of *S. birrea* does not form part of the traditional health practitioner's mixture. The leaf extract was prepared to compare the different plant parts as it was in abundance in the laboratory with its chemistry previously investigated and reported.^[65]

Table 2. 1: Identification of the collected plants and voucher specimen numbers of different plants collected.

Plant name	Plant part	Voucher number
<i>Sclerocarya birrea</i>	Stem and leaf	126589
<i>Gnidia sericocephala</i>	Roots	126590
<i>Senna italica</i>	Roots	126591
<i>Pentanisia prunelloides</i>	Roots	126592

2.8.2 Dichloromethane : methanol extraction of each of the four plants and the four plant mixture

S. birrea stem, *G. sericocephala* roots, *S. italica* roots and *P. prunelloides* were separately dried at 60 °C in an oven, drying time differed based on plant part. The dried plant material was grounded into fine powder using a hammer mill. The plants (2 g each) were extracted separately using a mixture of dichloromethane: methanol in a 1:1 ratio (20 ml). The extraction

was done by agitation with a stirrer bar for three hours and filtered under vacuum (Buchner funnel). For each plant, the extraction was repeated three times and the filtrates combined and evaporated to dryness using a rotary evaporator. The dried extracts were weighed and kept at a temperature of 4°C until biological analysis. The DCM : MeOH extract of the four plant mixture was prepared by mixing the individual plants in a 1:1:1:1 ratio under the same conditions describes above. The process of extraction is shown in Figure 2.7.

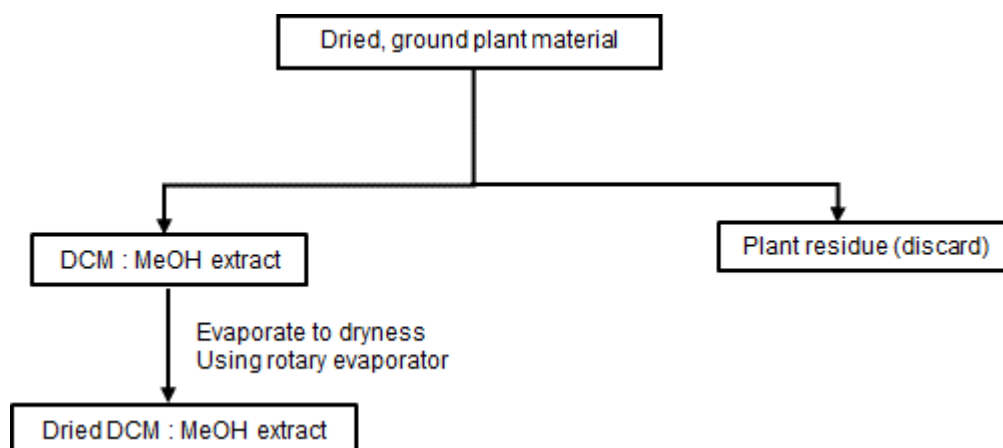


Figure 2. 7: Process for the extraction of individual plants and the four plant mixture using DCM : MeOH (1:1).

2.8.3 Extraction of the separate four plants and the four plant mixture using pharmaceutically accepted solvents.

The development of a traditional medicine requires the use of pharmaceutically accepted solvents such as water and ethanol.^[64] The dried and grounded 100 g of *G. sericocephala* roots, *S. italica* roots, *P. prunelloides* roots, *S. birrea* stems and the mixture of the four plants were extracted separately with 1 L of water, ethanol, 80% and 50% ethanol in water and agitated for 3 hours using a magnetic stirrer. The extracts were filtered under vacuum and dried using a rotary evaporator Figure 2.8.

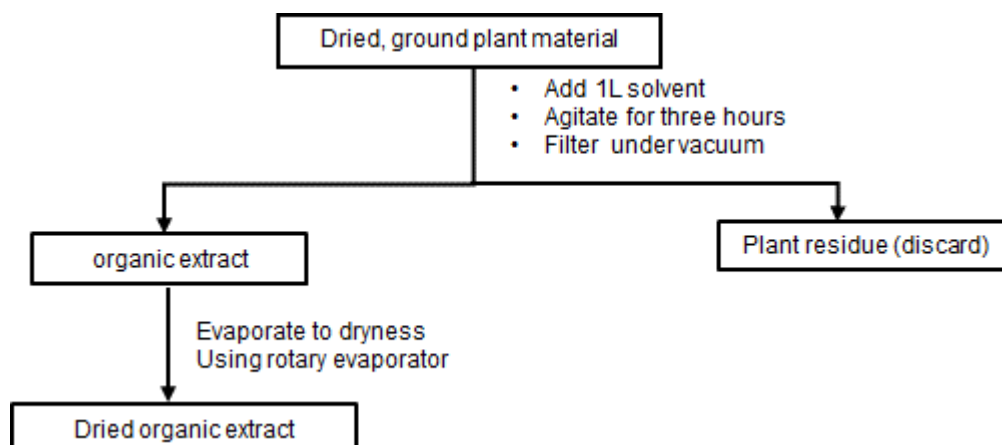


Figure 2. 8: Process for the separate extraction of individual plants and of four plant mixture using ethanol, 80% and 50% ethanol in water.

2.8.4 Sequential extraction of *Gnidia sericocephala* roots

Grounded roots of *G. sericocephala* (20 g) was sequentially extracted using hexane, dichloromethane, ethyl acetate and methanol. Extraction was performed by adding 200 ml of hexane to the plant material and agitating using a stirrer bar for three hours at room temperature. Extraction was repeated three times to achieve maximum extraction yield. The extract was then filtered under vacuum using Whatman No1 filter paper. The hexane plant residue was extracted with 200 ml of DCM and filtered. The DCM plant residue was extracted with 200 ml ethyl acetate (EtOAc) and filtered. The EtOAc plant residue was extracted with 200 ml methanol (MeOH) and filtered. All the extracts were separately evaporated to dryness using a rotary evaporator under reduced pressure and were weighed and stored in a cold room at 4 °C. Figure 2.9 shows the process of the sequential extraction.

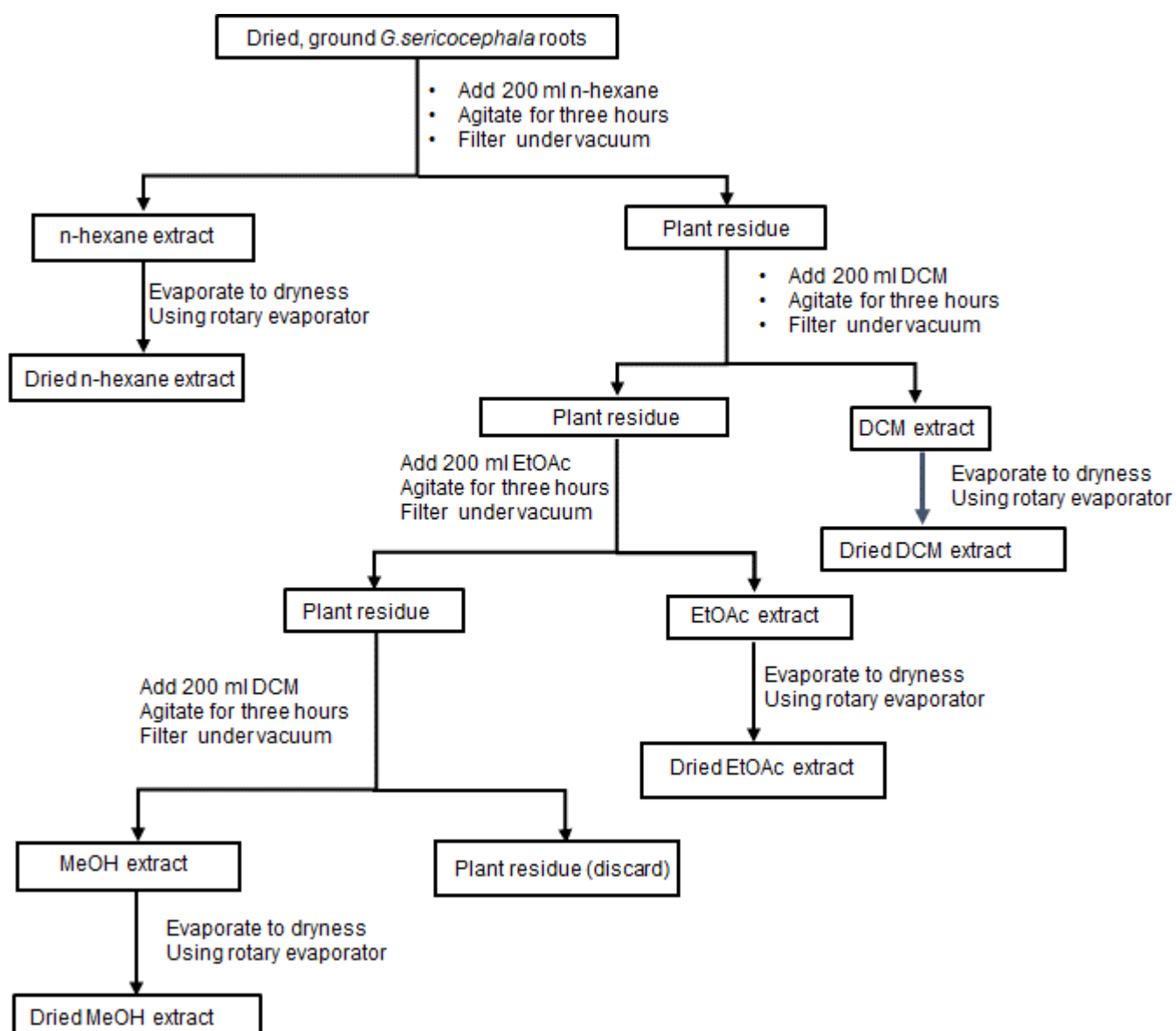


Figure 2. 9: Process describing the sequential extraction of *G. sericocephala* using non-polar to polar solvents.

2.8.5 Concentration of the active compounds in the ethanol extract of *G. sericocephala* and ethanol:water extract of the four plant mixture through liquid- liquid partitioning

The biologically active compounds in the ethanol extract of *G. sericocephala* were concentrated by partitioning the extract between different solvents to improve its biological activity. The extract (300 mg) was partitioned between deionised water and ethyl acetate as shown in Figure 2.10 by resuspending the extract in 60 ml deionised water and extracting with ethyl acetate (60 ml) three times in a separatory funnel. The combined organic layers were

separately concentrated to dryness using a Genevac Z-2 and weighed. The process was repeated for the four plant mixture

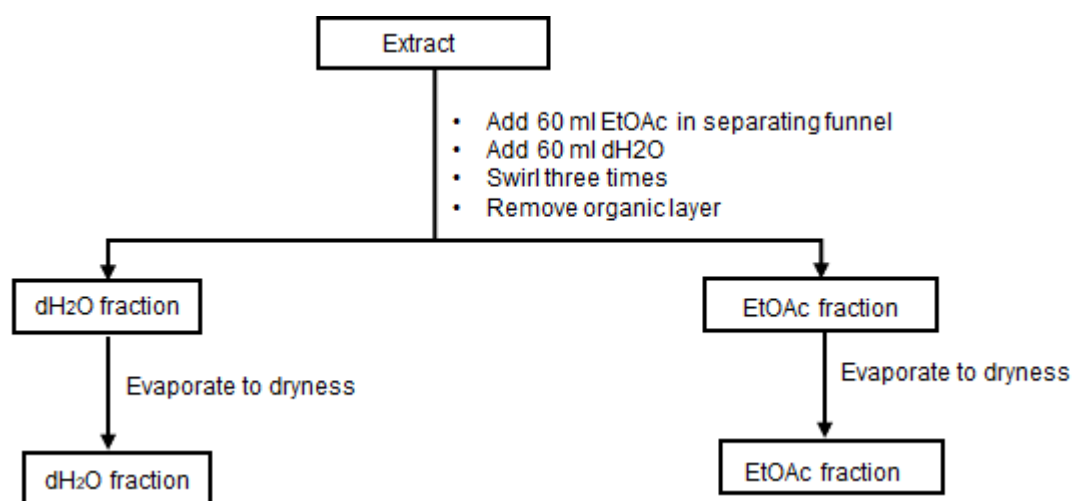


Figure 2. 10: Process for the concentration of the ethanol extract of *G. sericocephala* and the 50% ethanol extract of the four plant mixture through liquid-liquid partitioning between water and ethyl acetate

The ethanol extract (300 mg) was resuspended in 90% ethanol (20 ml) and extracted with hexane (10 ml) three times in a separatory funnel as shown in Figure 2.11. The hexane fractions were discarded and the 90% ethanol fraction dried and weighed. The procedure was repeated for the 50% ethanol extract of the four plant mixture.

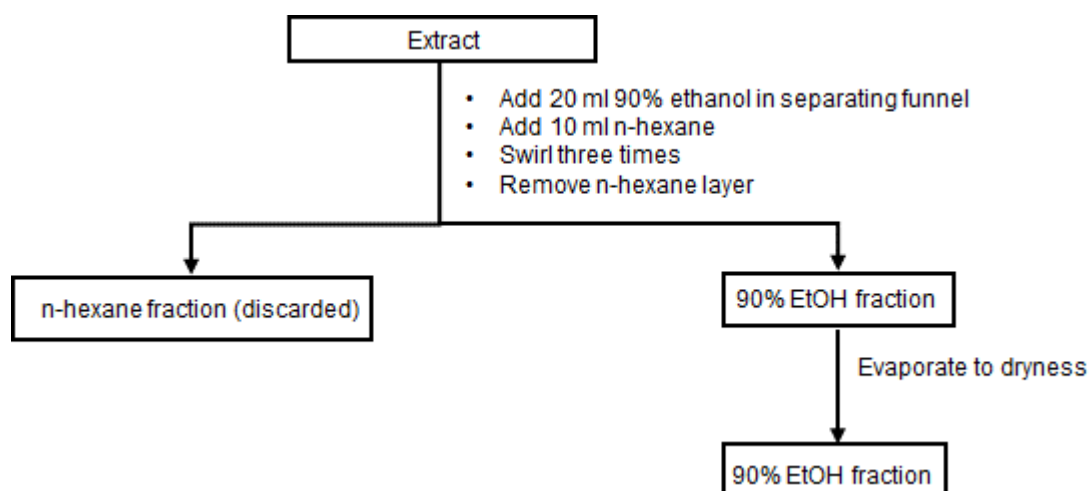


Figure 2. 11: Process for the concentration of the ethanol extract of *G. sericocephala* and the 50% ethanol extract of the four plant mixture through liquid-liquid partitioning between 90% ethanol and hexane.

2.9 Anti-HIV bio-assaying of extracts using the DeCiPhR assay

The anti-HIV assays were carried out at the University of Basel (UNIBAS) as part of a collaboration between the University of Pretoria and the University of Basel. Training in the bioassay techniques was done and conducted in Professor Thomas Klimkait's Research lab at the Department of Biomedicine supported by the European Virus Archive goes Global (EVAg) that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 653316. Samples were analysed in the Research lab at the Department of Biomedicine, UNIBAS, as part of the training.

All extracts were screened against HIV using pNL-NF as a CXCR4 tropic and pNL-BAL as a CCR5 tropic virus. The assay was carried out by transfecting human epithelioid cell line (293T cells) with plasmid DNA using jet prime as a transfecting agent. The transfected cells are then given a period of four days to replicate and spread in the absence or presence of the extracts by co-culture with a reporter cell line SXR5 and the virus. The reporter cell line SXR5 genome stably expresses HIV long terminal repeats (LTR) LacZ gene that reveals β -galactosidase activity. The activity of β -galactosidase is expressed after successfully infecting the cell line with HIV due to tat protein expressing the activation of LTR LacZ gene, which is used as a marker for viral replication. In the final step of the process, the cells are incubated with a chromogenic substrate for β -galactosidase, orthonitrophenyl-galactopyranoside (ONPG) and the colour development can be measured photometrically.^[54,66]

2.9.1 Transfection of 293 T cells

Transfection was carried out in a sterile 2 ml screw cap tube, where 3 μ g of plasmid DNA (pNL-NF or pNL-BaL) was added into 300 μ l of jetPRIME[®] buffer and mixed by vortexing. 6 μ l

of jetPRIME® reagent was added into the mixture, vortexed and incubated for 10 minutes at room temperature as shown in Table 2.2. 293T cells were harvested by trypsination and counted using a Neubauer chamber as shown in Table 2.3. Cell aliquots of 2.5×10^5 or 5.0×10^6 μ l in 500 μ l of complete Dulbecco's Modified Eagle Medium (DMEM) were added into the transfection mixture after the incubation period and the temperature was kept at 37 °C for 4 hours in 5% carbon dioxide. In an abgene 96-well microtiter plate 10 μ l of extracts of 20x dilutions in cold dimethoxymethane /dimethyl sulfoxide cDMME/DMSO were added.

Table 2. 2: Transfection of 293T cells

Per plate	1X 96 well plate	2X 96 well plate
Plasmid DNA	3 μ g	6 μ g
jetPRIME® buffer	300 μ l	600 μ l
jetPRIME® reagent	6 μ l	12 μ l

Table 2. 3: Addition of cells into transfection mix

Cell seeding for 4 days	1x 96 well plate	2x 96 well plate
293T cells	2.5×10^5 cfu/ μ l	5×10^5 cfu/ μ l
Adjust to	500 μ l	500 μ l
Final volume with transfection mix	800 μ l	800 μ l

2.9.2 Preparation of plant extract dilutions

A stock solution of 10 mg/ml was prepared by adding DMSO to the plant extracts and mixed well by vortexing. The initial concentration was diluted to 500 μ g/ml and 50 μ g/ml using a 20-fold serial dilution (Table 2.4). The two concentrations were further diluted from 500 μ g/ml to 25 μ g/ml and from 50 μ g/ml to 2.5 μ g/ml in 96-well plates.

Table 2. 4: Dilution used for the extracts

	Stock concentration (10 mg/ml)	Volume of extract dilution	Final volume in well	Final concentration in well
Deep well serial dilution	500 µg/ml 50 µg/ml	100 µl	(+ 900 µl for 1:10 dilution = 1 ml	20x
96 well triplicates	25 µg/ml 2.5 µg/ml	10 µl (Pre added)	(1+ 90 µl final mix)= 200 µl	1x

2.9.3 Addition of SXR5 cells

SXR5 cells were harvested by trypsinization, and cells were counted using a Neubauer chamber. An appropriate number of SXR5 cells were prepared in a 50 ml tube. The transfection mix was removed from 293T cells by centrifugation (150g for 5 minutes). The cells were washed once with cDMEM and the transfection was suspended in 1 ml medium and added to the SXR5 cells. The final mix was transferred from the 50 ml tube into 96-well plate reservoirs by distributing 190 µl into corresponding wells of the plate. The plates were incubated for 4 days at 37 °C, 5% CO₂.

2.9.4 Distribution of final mix to 96-well plates

The final mix from the 50 ml tube was transferred into deep well plate reservoir. A small amount, 190 µl of final mix was distributed into corresponding wells of the plates. The plates were incubated for four days in a biosafety level 3 (BSL3) lab for 4 days at 37 °C, 5% CO₂.

2.9.5 Dose response determination of extracts

The dose response of extracts and fractions from *G. sericocephala* and the mixture of the four plants were further screened at different test concentrations to determine their dose response

curve against plasmids pNL-NF and pNL-BaL. The extracts were screened by preparing a stock solution of 1000 µg/ml solution and a series of 10 concentrations ranging from 1000-2 µg/ml was prepared using a 2-fold serial dilution. Each test concentration was further diluted using a 20-fold to give a set of 10 concentrations ranging from 50- 0.1 µg/ml. The IC₅₀ of extracts and compounds was calculated using GraphPad Prism 5 (GraphPad Software, Inc).

2.9.6 Data analysis

The plates were read at 405 nm wavelength and the OD/FL results were converted into percent inhibition using the following formula:
$$\text{Inh X} = 100 - \left(\frac{(\text{ReadoutX} - \text{Readout0\%})}{(\text{Readout100\%} - \text{Readout0\%})} \times 100 \right),$$

Where: InhX –percent of inhibition; ReadoutX -absorbance of well containing substance 'X'; Readout0% -absorbance average of 6 wells containing cells incubated in reference inhibitor. Efavirenz was used as a known inhibitor and DMSO was used as a toxicity control on the viability control plate. Initially the extracts were screened at a dual point concentration in triplicates.

2.9.7 Cytotoxicity assay

The cytotoxicity effect of the extracts was determined under the same cell culture conditions used for the anti-HIV activity. However, the transfection mix with 293 T cells is replaced with cDMEM. After four days of incubation period, the cytotoxicity was determined.

2.9.8 Latency reversal activity of extracts

The latency reversal activity of the extracts, fractions and compounds was carried out at the Wistar Institute, USA as part of a collaboration between the research group of Prof Ian Tietjen and the University of Pretoria. The LRA of the extracts was done *in vitro* using a non-infectious (Δenv/Δnef) proviral genome. Where, J-Lat cells are treated with a known concentration of extracts and/or control (known LRA) for 24 hours. The proviral gene expression marker green

fluorescent protein (GFP) is used measured by flow cytometry. Simultaneously, the cell viability of the extracts is determined by the number of cells displaying normal forward and side scatter parameters in LRAs treated cells compared to culture treated with 0.1% DMSO control vehicle.^[53,54]

2.9.9 Latency reversal properties and cell viability of extracts (brief description)

See supplementary data 1 as provided for by Prof Ian Tietjen of the Wistar Institute, USA

2.9.10 Data Analysis

See supplementary data 1 as provided for by Prof Ian Tietjen of the Wistar Institute, USA

2.9.11 UPLC-QTOF-MS/MS analysis of the extracts

The chemical profile of active extracts was submitted for chemical fingerprinting to identify compounds responsible for the anti-HIV properties in the extracts. This was performed using a Waters Acquity UPLC system (Waters Corp., MA USA) equipped with a binary solvent delivery system. The auto sampler was operated using MassLynx 4.1 software (Waters Inc., Milford, Massachusetts, USA) to obtain the chemical profile of the crude extracts. Instrument calibration was performed using sodium formate solution over a mass range of 50-1200 Da to an absolute mass accuracy of <0.5 mDa.

The separation of compounds was performed on a Waters BEH C18 (1.7 µm) reverse phase column with particle size 2.1 mm × 100 mm. The mobile phase consisted of water in 0.1 % formic acid (solvent A) and methanol in 0.1 % formic acid (solvent B). The gradient elution was carried as follows: 3% (0-1 min) and was kept constant at 100% B between (0.3-14 and 14-16 min) and later optimized to 3% B (16.50-20.00 min). The flow rate was maintained at a 0.4 ml/min with an injection volume of 5µl with a constant column temperature of 40 °C. All the extracts were prepared to a final concentration of 1 mg/ml in 80% acetonitrile and were run in both positive and negative ionisation mode. Elemental composition, MS/MS fragmentation

patterns, iFit values, mass error, Metlin, Mass bank, ChEBI (chemical entities of biological interest) and Metfusion were used for the tentative identification of compounds.

2.10 Results and discussion

2.10.1 Extraction yields of all extracts

The DCM : MeOH extraction was carried out to obtain extracts with both more polar and non-polar compounds. The DCM : MeOH extract yields of the four separate plants and the four plant mixture is shown in Table 2.5. The DCM : MeOH extract of the leaves of *S. birrea* had the highest yield of 18.5% while the roots of *S. italica* had the lowest yield of 3.15%. The DCM : MeOH extract of the four plant mixture gave a yield of 5.97%.

Table 2. 5: Percentage yields of DCM : MeOH extracts

Plant name	Plant part	%Yield (w/w)
<i>G.sericocephala</i>	Roots	3.99
<i>S. italica</i>	Roots	3.15
<i>S. birrea</i>	Stems	7.9
	Leaves	18.5
<i>P. prunelloides</i>	Roots	3.52
Four plant mixture	Roots and stems	5.97

The use of the sequential extraction using non-polar and polar solvents was done to separate the polar compounds from the non-polar compounds. The percentage yield of extracts shows an increase in extract yield with an increase in solvent polarity as the hexane extract had the lowest yield of 0.54% and methanol with the highest extract yield of 10.84% as shown in Table 2.6.

Table 2. 6: Percentage yield of extracts obtained by sequential extraction.

Extract type	%Yield (w/w)
n-Hexane	0.54
DCM	1.24
EtOAc	1.59
MeOH	10.84

The use of ethanol, 80% ethanol and 50% ethanol in water was carried out for the development of the ingredient using pharmaceutically accepted solvents while retaining or improving the activity of the plants. The percentage yield of *G. sericocephala* extracts and the four plant mixture of the shown in Table 2.7. The 50% ethanol extract had a yield of 18.25 % (w/w), the 80% ethanol extract a yield of 7.32%, (w/w) and the ethanol extract a had a yield of 6.98%, (w/w). The 50% ethanol solvent mixture gave the highest extraction yield, ethanol and the 80% ethanol gave similar lower yields. The different extracts of the four plant mixture had a percentage yield of ethanol (18.32, w/w), 80% ethanol (21.54, w/w) and 50% ethanol (18.25,w/w)

Table 2. 7: Percentage yield of extracts obtained using pharmaceutically acceptable solvents

Plant	Extract type	% Yield
<i>G. sericocephala</i>	EtOH	6.98
	80% EtOH	7.32
	50% EtOH	18.26
	H ₂ O	25.36
Four plant mixture	EtOH	18.32
	50% EtOH	18.25
	80% EtOH	21.54
	H ₂ O	--

2.10.2 Extraction yields of fractions prepared through liquid-liquid partitioning

The liquid-liquid partitioning of the *G.sericocephala* ethanol extract between water and ethyl acetate resulted in 36.24% (w/w) yield for ethyl acetate fraction and 28.86% yield for the water fraction as shown Table 2.8. The further separate partitioning of the ethanol extract between 90% ethanol and hexane resulted a 95.25% (w/w) yield of the 90% ethanol fraction.

Table 2. 8: Fraction yields (%) obtained from the liquid-liquid partitioning of the ethanol extract of *G. sericocephala*

Fraction type	%Yield (w/w)
EtOAc	36.24
H ₂ O	28.86
ethanol 90%	95.25

The percentage yields of the fractions from the four plant mixture is shown in Table 2.9. The liquid-liquid partitioning of the 50% ethanol extract of the four plant mixture between the ethyl acetate and water resulted in a yield of 23.62% (w/w) of the ethyl acetate fraction and 32.20% of the water fraction. The liquid-liquid partitioning of the four plant mixture extract between the 90% ethanol and hexane resulted in a yield of 28.75% of the 90% ethanol fraction.

Table 2. 9: Fraction yields (%) obtained through liquid-liquid partitioning of 50% ethanol extract of the four plant mixture

Fraction type	%Yield (w/w)
EtOAc	23.62
H ₂ O	32.24
90% EtOH	28.80

2.10.3 Anti-HIV activity of extracts

2.10.3.1 Anti-HIV activity of DCM : MeOH extracts

The anti-HIV (pNL-NF plasmid) activity of the six DCM : MeOH extracts tested in transfected 293T cells and reporter cells SXR5 at dual concentrations of 25 and 2.5 µg/ml in triplicate is shown in Table 2.10. The *G. sericocephala* root extract and the four plant mixture extract were the only extracts which showed inhibition of HIV replication at both test concentrations with limited cytotoxicity. A >50% inhibition was considered promising for further research. The *G. sericocephala* and the four plant mixture extracts exhibited anti-HIV activity at the lower concentration of 2.5 µg/ml by $112.8 \pm 7.0\%$ and $69.5 \pm 6.5\%$, respectively.

Table 2. 10: *In vitro* anti-HIV and cytotoxicity activity of DCM : MeOH extract against pNL-NF plasmid tested at 25 µg/ml and 2.5 µg/ml in the deCIPhR assay.

Extracts	25 µg/ml		2.5 µg/ml	
	%inhibition±	% toxicity±	%inhibition±	% toxicity±
	Std. Dev	Std. Dev	Std. Dev	Std. Dev
<i>S. birrea</i> stems	19.0±23.0	98.5±3.1	-112.3±9.4	-0.2±3.3
<i>s. birrea</i> leaves	131.5±1.9	8.7±0.8	-103.4±12.2	5.1±8.8
<i>S. italica</i> roots	-246.9±25.7	19.3±2.1	-66.8±25.5	4.4±1.3
<i>G. sericocephala</i> roots	122.7±2.2	7.2±2.0	112.8±7.0	25.4±6.3
<i>P. prunelloides</i> roots	-109.9±18.5	21.7±1.8	-36.5±29.2	8.0±1.7
Four plant mixture	122.8±4.2	7.5±2.0	69.5±6.5	16.9±2.8
Efavirenz	100±0.0	0	100±0.0	0

The current study showed that the extracts from the individual plant *S. birrea*, *S. italica*, *P. prunelloides* did not possess anti-HIV (pNL-NF) activity in the deCIPhR assay . Consequently, the anti-HIV activity that was found in the mixture of the four plants may have been due to the presence of compounds from *G. sericocephala* or the synergistic effects of compounds when in a mixture.

2.10.3.2 Concentration response studies of DCM : MeOH extracts from *Gnidia sericocephala* and the four plant mixture against HIV plasmids pNL-NF and pNL-BaL.

The anti-HIV activity of the DCM : MeOH extract of *G. sericocephala* when screened against the plasmid pNL-NF is shown in Figure 2.12. The extract showed inhibition activities of $103.7 \pm 2.01\%$, $100.1 \pm 4.4\%$, $108.3 \pm 0.7\%$, $106.7 \pm 0.8\%$, $88.50 \pm 2.3\%$ and $55.21 \pm 4.4\%$ at concentrations 50, 25, 12.5, 6.3, 3.1 and 1.6 $\mu\text{g/ml}$, respectively. The extract when tested against the HIV plasmid pNL-BaL exhibited inhibition activities of $59.6 \pm 6.0\%$ at 50 $\mu\text{g/ml}$, $77.9 \pm 3.5\%$ at 25 $\mu\text{g/ml}$, $93.3 \pm 2.4\%$ at 12.5 $\mu\text{g/ml}$, $87.5 \pm 3.0\%$ at 6.3 $\mu\text{g/ml}$ and $62.9 \pm 4.2\%$ at 3.1 $\mu\text{g/ml}$. The extract was inactive against pNL-BaL at concentrations less than 3.1 $\mu\text{g/ml}$.

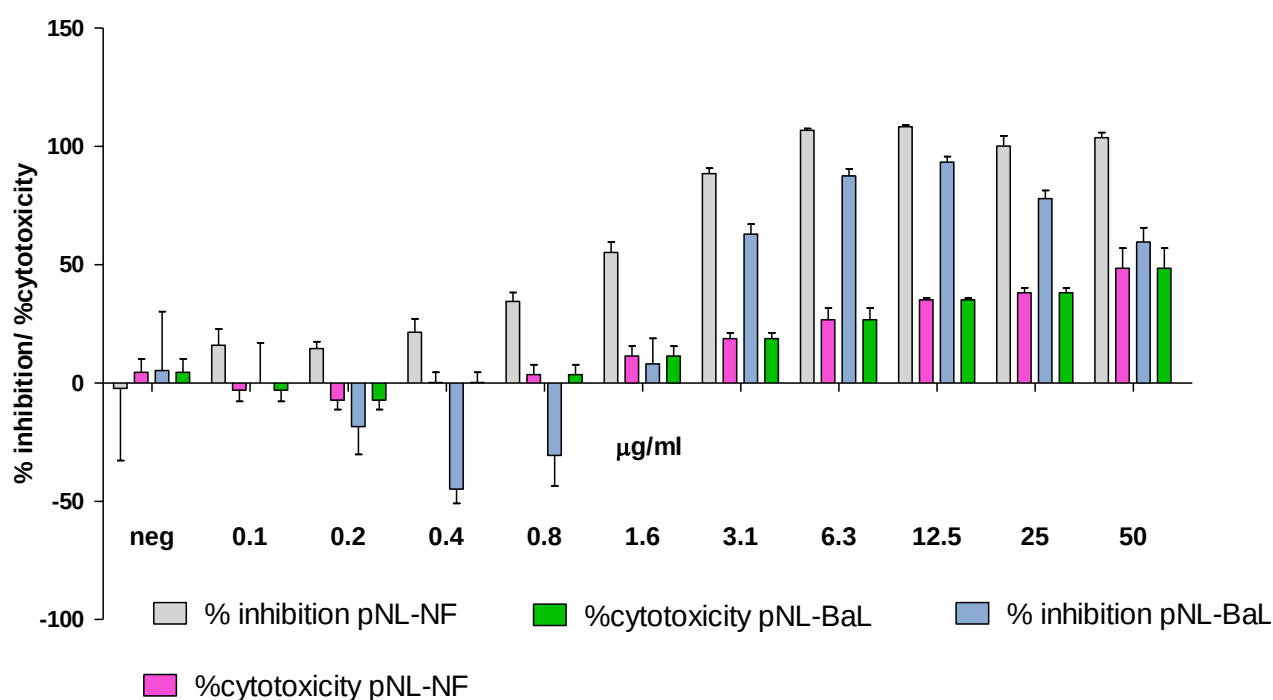


Figure 2.12: Anti-HIV activity and cytotoxicity levels of DCM : MeOH extract of *G. sericocephala* against the pNL-NF and pNL-BaL plasmids.

The IC_{50} of the DCM : MeOH extract of *G. sericocephala* against the pNL-NF plasmid was determined as 1.67 $\mu\text{g/ml}$ while against the plasmid pNL-BaL it was 1.98 $\mu\text{g/ml}$ as shown in Figures 2.13 and 2.14, respectively. The DCM : MeOH extract of *G. sericocephala* showed higher inhibition activities against the HIV (pNL-NF) plasmid, as it exhibits anti-HIV activities

of greater than 80% at 5 test concentrations when compared to HIV (pNL-BaL) plasmids. The extract showed effectiveness for both viral plasmids with varying anti-HIV activities at individual test concentrations. There is not much difference in the concentration-response curves between the two plasmids .

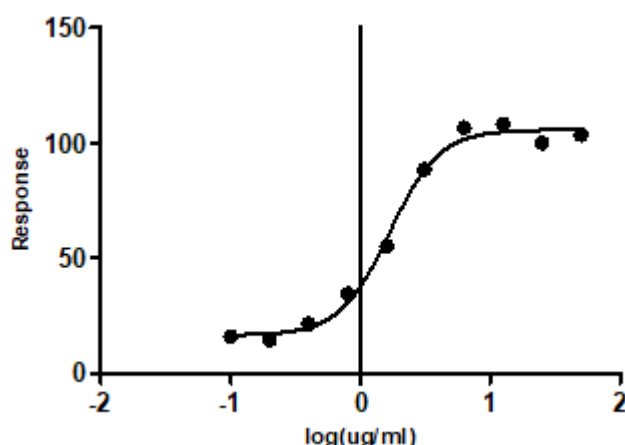


Figure 2.13: Concentration-response curve of *G. sericocephala* DCM : MeOH extract against HIV pNL-NF plasmid. The extract had an IC_{50} of 1.67 $\mu\text{g/ml}$.

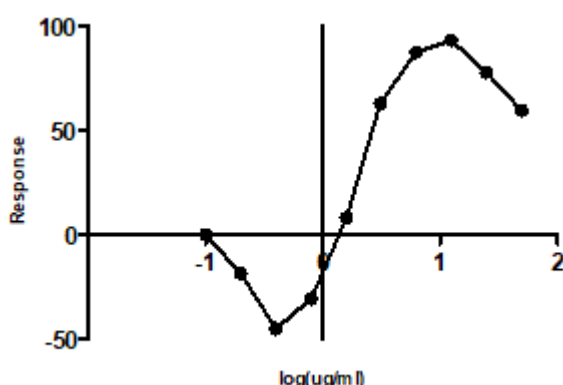


Figure 2. 14: Concentration-response curve of *G. sericocephala* DCM : MeOH extract against HIV pNL-BaL plasmid. The extract had an IC_{50} of 1.98 $\mu\text{g/ml}$.

The DCM : MeOH extract of the four plant mixture exhibited anti-HIV activities at the higher test concentrations when tested against plasmid pNL-NF with inhibition activity of $97.4 \pm 4.9\%$ at 50 $\mu\text{g/ml}$, $97.1 \pm 6.0\%$ at 25 $\mu\text{g/ml}$, $91.8 \pm 6.4\%$ at 12.5 $\mu\text{g/ml}$ and $68.2 \pm 10.0\%$ at 6.3 $\mu\text{g/ml}$ as shown in Figure 2.15. The mixture did not possess any anti-HIV activities at the lower test concentrations against this plasmid. In contrast to pNL-NF, the extract only showed anti-HIV activity at three high test concentrations against plasmid pNL-BaL which were $79.9 \pm 11.4\%$ at 50 $\mu\text{g/ml}$, $76.1 \pm 7.7\%$ at 25 $\mu\text{g/ml}$ and $56.8 \pm 17.7\%$ at 12.5 $\mu\text{g/ml}$ (green bars). The extract,

at lower concentration showed secondary activity that may be attributed to its negative inhibition. This behaviour can be assigned to the extracts ability to induce the HIV long terminal repeat promoter. The extract in higher test concentrations showed good anti-HIV activity for both receptors. However, the extract was mainly potent against the pNL-NF plasmid (encoding for CXCR4 receptor) at different concentrations as it also showed activity up to 6.3 $\mu\text{g/ml}$ (pink bars). The plasmid pNL-BaL (encoding for CCR5 receptor) is usually detected in late stage of viral replication.^[67] This indicates that the DCM : MeOH extract of the four plant mixture was mainly effective for inhibiting viral strains with pNL-NF plasmid. The IC_{50} values of the DCM : MeOH extract from the four plant mixture against the two plasmids was determined as 2.46 $\mu\text{g/ml}$ for plasmid pNL-NF and 4.84 $\mu\text{g/ml}$ for pNL-BaL plasmid as shown in Figures 2.16 and 2.17, respectively.

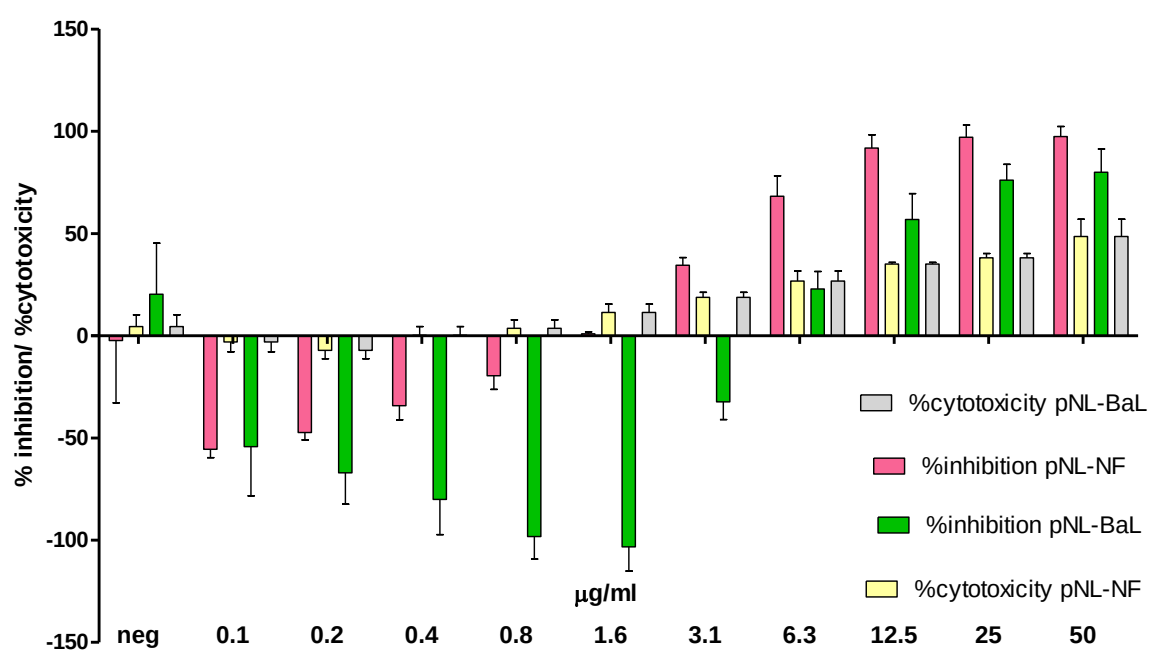


Figure 2.15: Anti-HIV activity of the four plant mixture extract against pNL-NF and pNL-BaL plasmids

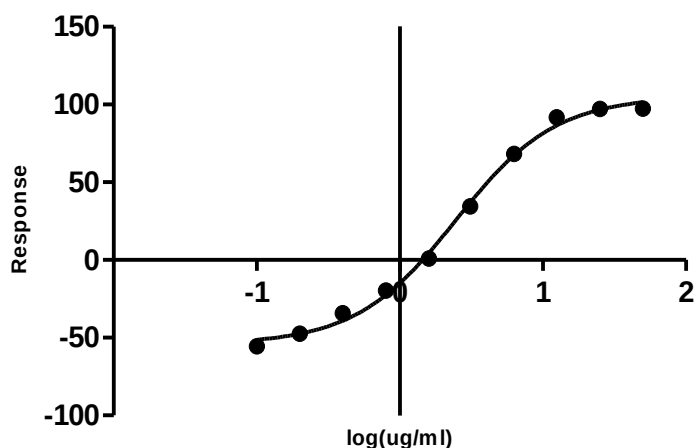


Figure 2.16: Concentration response curve of the four plant mixture DCM : MeOH extract against pNL-NF plasmid. The extract had an IC_{50} of 2.46 $\mu\text{g/ml}$.

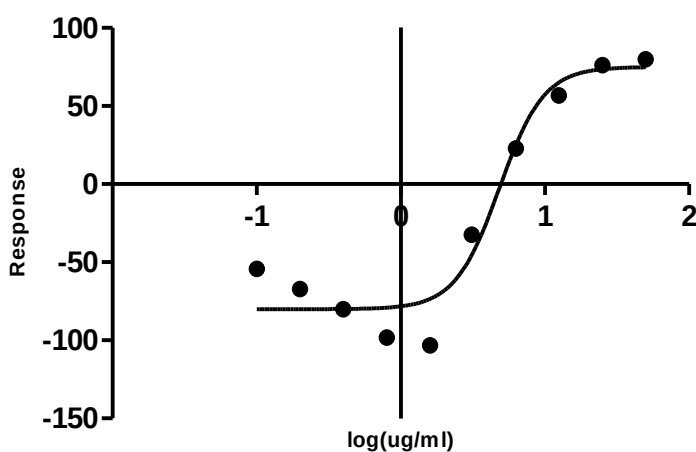


Figure 2.17: Concentration response curve of the four plant mixture DCM : MeOH extract against pNL-BaL plasmid. The extract had an IC_{50} of 4.84 $\mu\text{g/ml}$.

Plant species from the *Thymelaeaceae* family including *Gnidia* species^[39,44] have been shown to exhibit excellent anti-HIV activity. The anti-HIV-BaL *in vitro* activity of the ethyl acetate extract of *Thymelaea hirsuta* (*Thymelaeaceae*) was reported to have showed an IC_{50} value of $0.8 \pm 0.002 \mu\text{g/ml}$.^[68] Also, Vidal *et al.*, (2013) reported the anti-HIV *in vitro* activity of the dichloromethane extract of *Daphne gnidium* (*Thymelaeaceae*) with an IC_{50} value of 280 ng/ml.^[39] Additionally, Li *et al.* (2014) reported the petroleum ether extract of the flower buds of *Daphne genkwa* to exhibit good HIV inhibition activity with an IC_{50} value of $0.083 \mu\text{g/ml}$.^[69] The anti-HIV activity shown by these extracts is in support of the anti-HIV activity shown by

G. sericocephala, especially as the extract type indicated the active compounds are in all likelihood, are relatively non-polar as in all cases the activity was located in extracts prepared from non-polar to medium polar solvents.

The DCM : MeOH extract of *G. sericocephala* contained polar and non-polar compounds making the extract complex to isolate and identify compounds. In order to simplify the chromatography, the plant was extracted sequentially with hexane, dichloromethane, ethyl acetate and methanol to group compounds based on their polarity to localise the activity. The good anti-HIV activity portrayed by *G. sericocephala* when compared to the anti-HIV activity shown by the four-plant mixture may have been due to these compounds in the mixture in lower concentrations as they are diluted by other plant extracts. Based on the data, *G. sericocephala* extracts portrayed the best anti-HIV activity and warranted further studies to isolate and identify the active ingredients.

2.10.3.3 The anti-HIV activity of *Gnidia sericocephala* of sequentially prepared extracts

The anti-HIV activity of the four extracts from *G. sericocephala* prepared by sequential extraction were screened against HIV and the results recorded after 30 minutes of incubation is shown in Figure 2.18.

The hexane extract showed inhibition of $81.6 \pm 7.6\%$ at the lowest test concentration of 0.8 $\mu\text{g/ml}$, while the dichloromethane fraction showed inhibition activities at all test concentrations with $113.8 \pm 18.9\%$ at the lowest of concentration of 0.8 $\mu\text{g/ml}$. The dichloromethane extract showed comparable inhibition with the positive control efavirenz. These results also indicated that the compounds responsible for the anti-HIV activity are medium to non-polar and are in agreement with the results shown by other extracts from the *Thymelaeaceae* species. The ethyl acetate extract showed inhibition activities of $124.5 \pm 2.2\%$ at test concentration of 7.4 $\mu\text{g/ml}$ and was inactive below this test concentration, while the methanol extract showed inhibition activities of $103.6 \pm 7.3\%$ and was inactive at test concentrations lower than this.

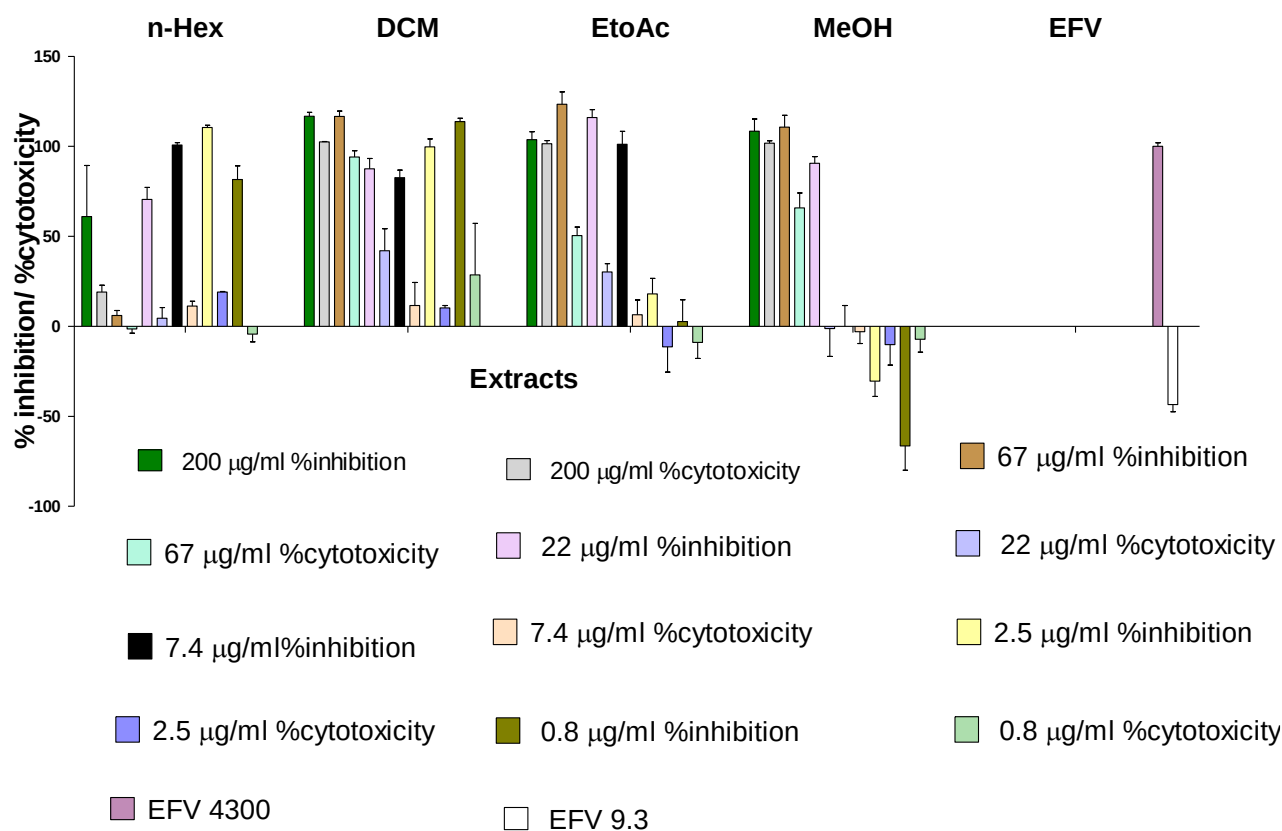


Figure 2.18: The anti-HIV activity of sequential extracts of *G. sericocephala* after 30 minutes incubation and positive control efavirenz. n-Hex (hexane), DCM (dichloromethane), EtOAc (ethyl acetate) and MeOH (methanol), EFV 4300 (efavirenz at 4300 nM) and EFV 9.3 (efavirenz at 9.3 nM).

The anti-HIV activity of the four extracts from *G. sericocephala* prepared by sequential extraction were screened against HIV and the results recorded after 4 hours of incubation is shown in Figure 2.19. The hexane extract showed inhibition activity of $77.8 \pm 5.3\%$ at the lowest test concentration of $0.8 \mu\text{g/ml}$, while the dichloromethane fraction showed inhibition activities at all test concentrations with $134.2 \pm 2.6\%$ at the lowest test concentration of $0.8 \mu\text{g/ml}$. The ethyl acetate extract showed inhibition activities of $103.6 \pm 7.3\%$ at test concentration of $7.4 \mu\text{g/ml}$ and was inactive at test concentrations lower than this, while the methanol extract showed inhibition activities of $89.9 \pm 12.0\%$ at test concentration of $22 \mu\text{g/ml}$ and inactive at test concentrations lower than this.

The good anti-HIV activity portrayed by the DCM extract indicates that the extract contains compounds that may be used as inhibitors of the late stages of viral replication as it exhibited effectiveness of up to 100% after 4 hours.^[67] The results shown by the hexane and DCM extracts after 30 minutes of incubation are an indication that both extracts may be used as viral entry inhibitors. The DCM extract shows consistent anti-viral activity in both time intervals and needs to be further investigated for its mode of action.

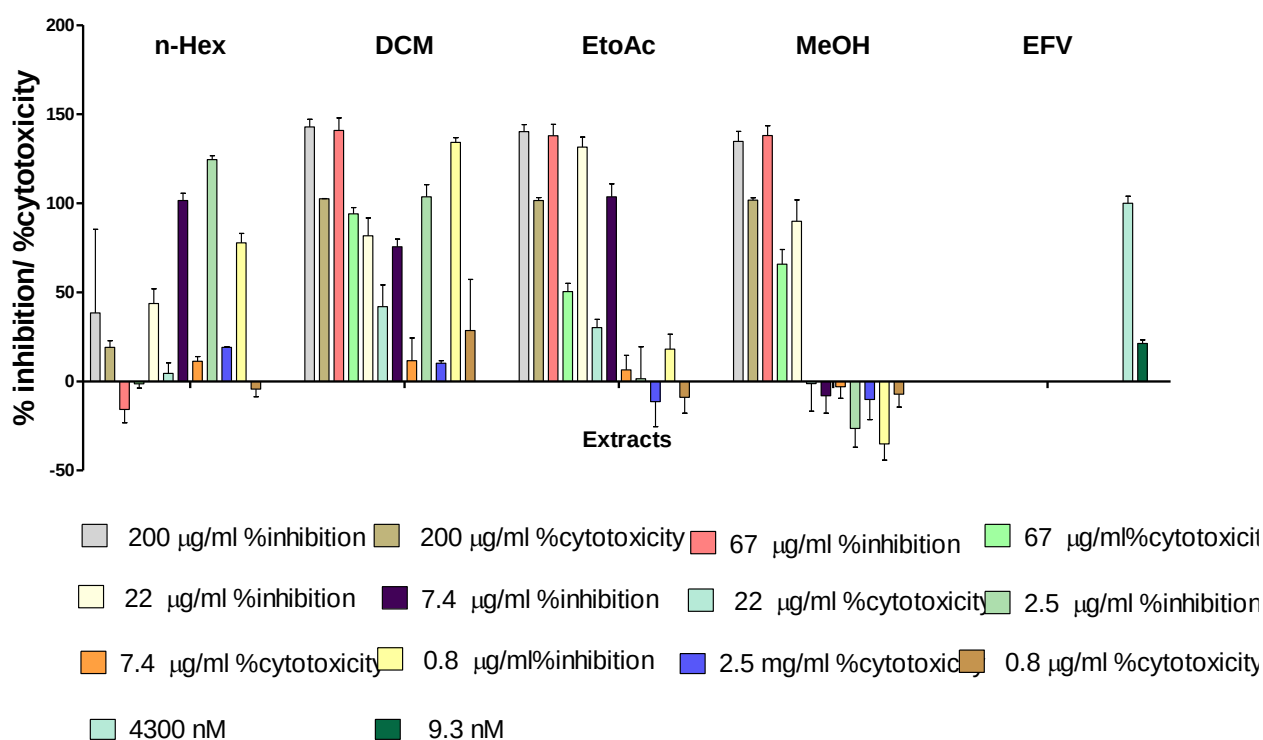


Figure 2.19: The anti-HIV activity of sequential extracts of *Gnidia sericocephala* after 4 hours of incubation and positive control efavirenz. n-Hex (hexane), DCM (dichloromethane), EtOAc (ethyl acetate) and MeOH (methanol), EFV 4300 (efavirenz at 4300 µM) and EFV 9.3 (efavirenz at 9.3 µM).

The anti-HIV properties of compounds from the *Thymelaeaceae* family to which *Gnidia* belongs have been reported.^[41] *Trigonostemon thyrsoides* of the *Thymelaeaceae* family is reported to contain compounds with anti-HIV properties with IC_{50} values ranging from 0.015 to 0.001nM and a therapeutic index (TI) ranging between 1618-17,619.^[70] Many daphnane diterpenoids known to occur in *Gnidia* species of the *Thymelaeaceae* family are reported to

exhibit moderate to good anti-HIV activities and some are said to possess latency reversal properties.^[70,71] The anti-HIV activity of the compounds from plants belonging in this family are reported to possess inhibitory effects against T-20 resistance strains with IC₅₀ values of approximately 10 ng/ml, suggesting that the compounds could be further developed as entry inhibitors.^[68,72,73] The dichloromethane extract of *G. sericocephala* was further chemically characterized using UPLC-QTOF-MS to identify the class of compounds responsible for the anti-HIV activity.

2.10.3.4 Anti-HIV activity of *G.sericocephala* and the four plant mixture of extracts using pharmaceutically accepted solvents

The ethanol and water extracts of *G. sericocephala* were screened for their anti-HIV activity at dual point test concentration of 2.5 µg/ml and 0.25 µg/ml and the results are shown in Figure 2.20. The water extract showed minimal inhibition of the virus as it only decreased viral replication by only 16.9±5.7% at 2.5 µg/ml and 11.7±30.9 at 0.25 µg/ml. The ethanol extract showed anti-HIV activity of 89.0±4.1% with limited cytotoxicity of 7.4±14.5% at 2.5 µg/ml and was however inactive at 0.25 µg/ml. The extract when screened at 2.5 µg/ml showed inhibition activity comparative to the positive control efavirenz at 92 nM. The ethanol extract was chosen as the most suitable extract for the development of the plant as a treatment for HIV.

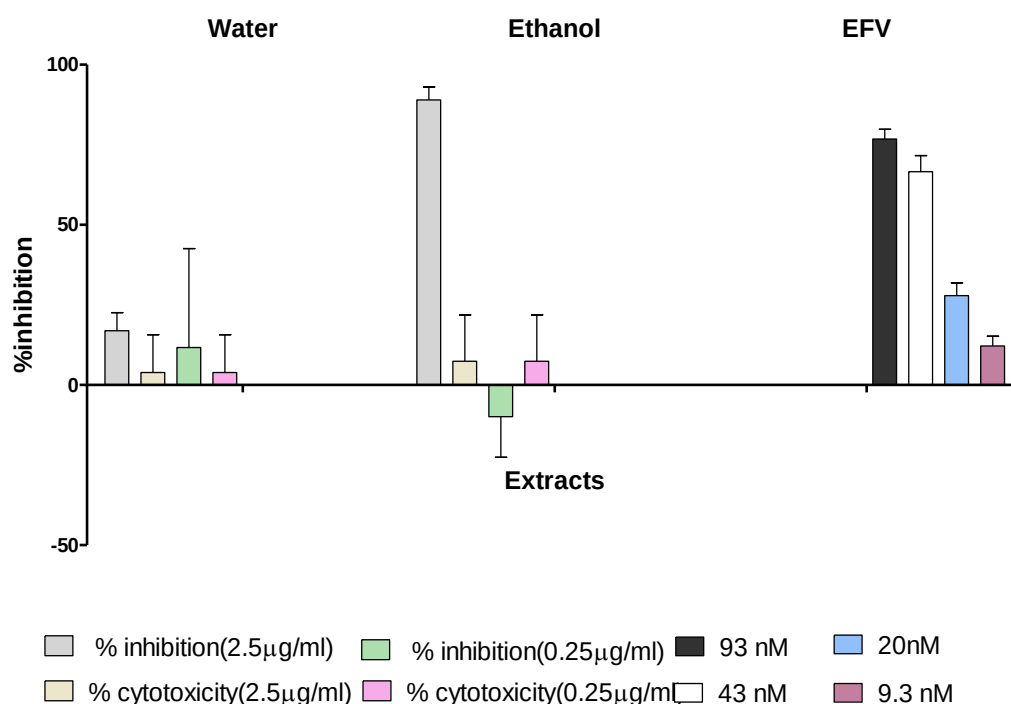


Figure 2.20: Anti-HIV activity of water and ethanol extracts of *G. sericocephala* and efavirenz (EFV) as positive control.

Previous studies on aqueous, alcoholic and hydro-ethanolic extracts of *Daphne gnidium* and *Euphorbia hirta* (*Euphorbeaeceae* and *Thymelaeaceae*) species were reported to possess anti-HIV activity.^[74] The phytochemicals present in these plants were reported to be daphnane diterpenoids such as gnididin, gniditrin, gnidicin and excoecariatoxin.^[39] The aqueous extract of *G. sericocephala* in this study did not possess any anti-HIV activity, whereas the ethanol extract showed good anti-HIV activity. The inactivity of the aqueous extract compared to the good anti-HIV activity of the ethanol extract of *G. sericocephala* may be attributed to the daphnane diterpenoids not being extracted in the water layer due to their polarity.

The ethanol, 80% ethanol and 50% ethanol extracts of *G. sericocephala* were screened against HIV at different concentrations (5, 1, 0.3 and 0.03 µg/ml) and the results are shown in Figure 2.21. The 50% ethanol extract showed anti-HIV activity of 97.4 ± 2.6 and cytotoxicity of $35.5 \pm 19.8\%$ at 5 µg/ml while it did not possess anti-HIV activity at the lower test concentrations. The inactivity portrayed by the extract at low test concentrations may be due

to the inactive polar compounds extracted with water resulting in the dilution of the active ingredients. The cytotoxicity of the extract in high test concentrations may be attributed to other toxic compounds extracted with this solvent mixture. These results deemed the 50% ethanol an unsuitable choice of solvent. The 80% ethanol extract showed anti-HIV activity of $101.7 \pm 3.5\%$ with cytotoxicity of $53.4 \pm 9.3\%$ at $5 \mu\text{g/ml}$. The high cytotoxicity levels at this test concentration may be due to the toxic compounds extracted using this solvent mixture. At low test concentrations of $1 \mu\text{g/ml}$, the 80% ethanol extract inhibited the virus by 89.1 ± 9.1 with minimal cytotoxicity of $11.9 \pm 1.1\%$. The ethanol extract showed anti-HIV activity of $97.5 \pm 4.8\%$ with cytotoxicity of $35.5 \pm 3.8\%$ at $5 \mu\text{g/ml}$ while at $1 \mu\text{g/ml}$ the extract inhibited the virus by $100.9 \pm 1.5\%$ and showed cytotoxicity of $20.5 \pm 4.7\%$. At the lowest test concentration of $0.3 \mu\text{g/ml}$ the extract inhibited the virus by $64.1 \pm 2.32\%$ with limited cytotoxicity and as this extract was the only extract which showed anti-HIV activity at the lowest test concentration, the extract was chosen for further development.

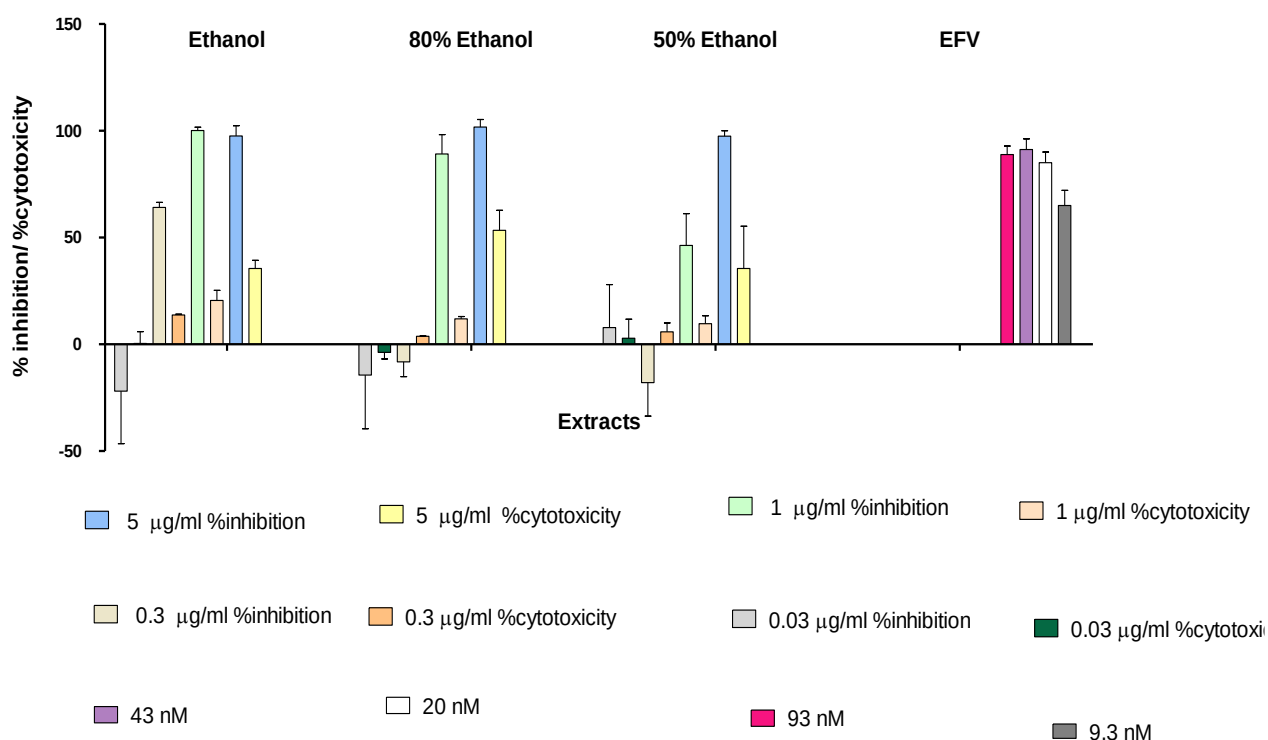


Figure 2.21: Anti-HIV activity and cytotoxicity of the hydro-ethanolic extracts (ethanol, 80% ethanol and ethanol 50%) of *G. sericocephala* roots. Efavirenz (EFV) used as positive control at 93 nM, 43 nM, 20 nM, and 9.3 nM.

The anti-HIV activity of the hydro-ethanolic extracts of the four-plant mixture was tested at four different concentrations (5, 1, 0.3 and 0.03 µg/ml) and the results are shown in Figure 2.22. All extracts showed a decrease in viral replication that was greater than 75% at 5 µg/ml. At 1 µg/ml only the ethanol and 50% ethanol extracts showed some decrease in the viral replication. At the lowest test concentration, all extracts were inactive. The ethanol and 80% ethanol extracts were unsuitable for continuous work due to the cytotoxicity they showed ($36.66 \pm 16.14\%$ and $38.08 \pm 6.2\%$), respectively. The 50% ethanol extract was selected as the most suitable hydro-ethanolic extract for the four plant mixture, as it showed minimal cytotoxicity (< 20%).

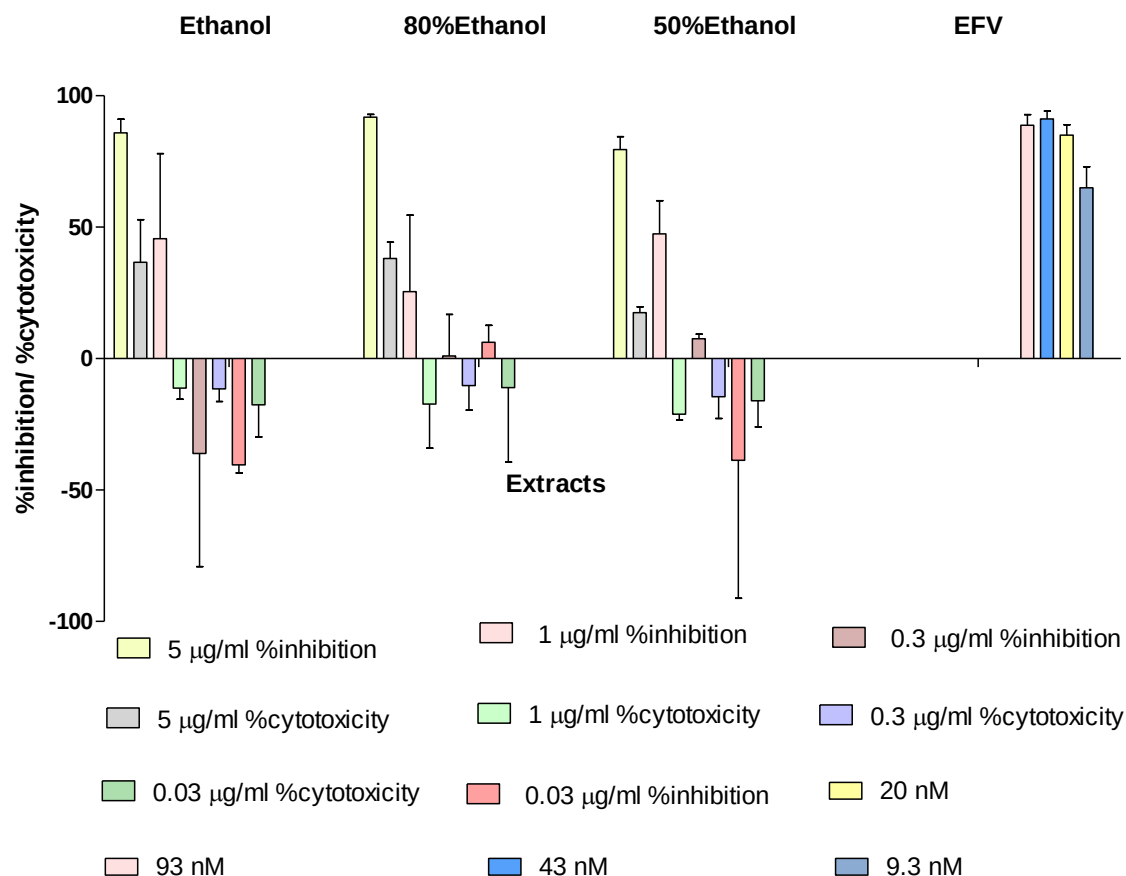


Figure 2.22: Anti-HIV activity of hydro-ethanolic (ethanol, 80% ethanol and ethanol 50%) extracts of the four plant mixtures.

The good anti-HIV activity showed by the 50% ethanol extract of the four-plant mixture and the ethanol extract of *G. sericocephala* led to further development and phytochemical analysis of the two extracts using UPLC-QTOF-MS.

2.10.3.6 The anti-HIV activity of fractions obtained through liquid-liquid partitioning of the ethanol extract of *G. sericocephala*

The purpose of the study was to determine the most active fractionation from the plant for its further development and to isolate and identify the compounds responsible for the anti-HIV activities. The anti-HIV activity of *G. sericocephala* fractions prepared through the liquid-liquid partitioning from the ethanol extract of the plant is shown in Figure 2.23. The 90% ethanol fraction obtained through liquid-liquid partitioning between 90% ethanol and hexane exhibited

anti-HIV with cytotoxicity. The 90% ethanol fraction showed anti-HIV activity of $105 \pm 1.1\%$, $86.5 \pm 3.4\%$ and $96.7 \pm 7.2\%$ when screened at 5, 2.5, 1.25 $\mu\text{g/ml}$, respectively. The ethyl acetate fraction showed inhibition activities greater than 50% at 0.25 $\mu\text{g/ml}$ with cytotoxicity. The cytotoxicity of the ethyl acetate and 90% ethanol fractions can be due to the highly concentrated nature of the active compounds in them. Based on these the ethyl acetate fraction of *G.sericocephala* was chosen as the best fraction for further developments as it showed inhibition activities comparable to efavirenz.

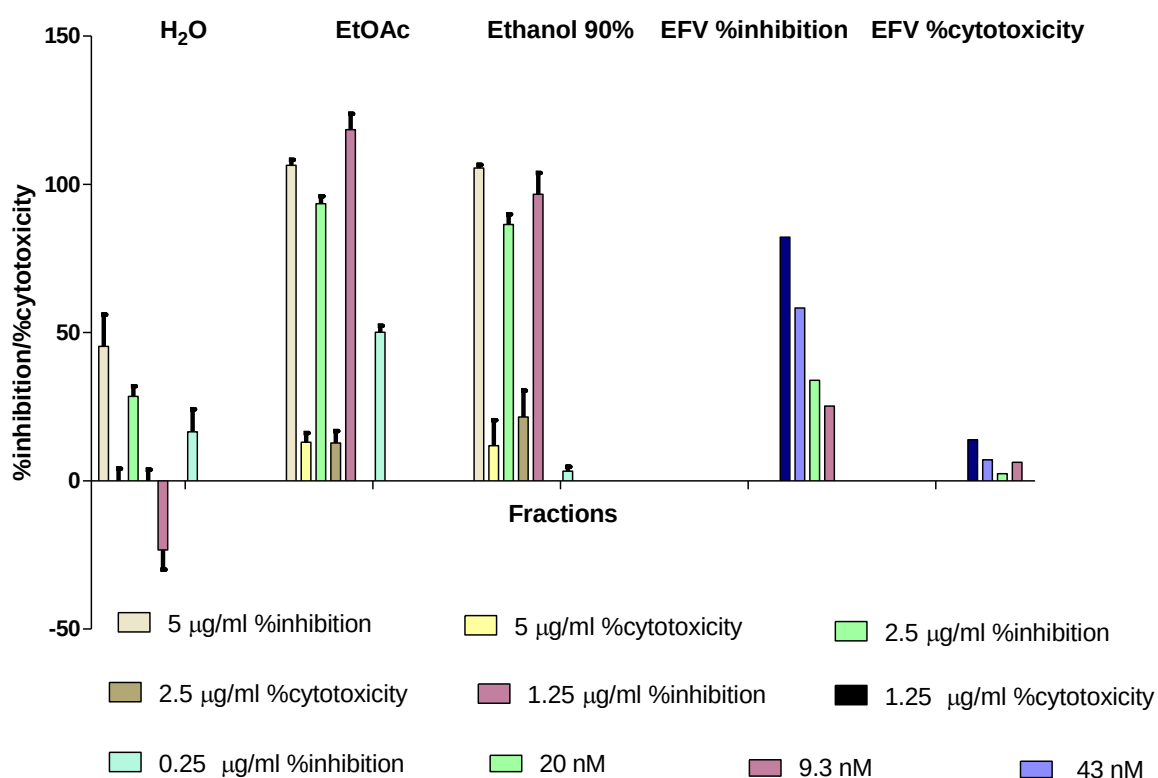


Figure 2.23: Anti-HIV activity of concentrated fractions from *G. sericocephala* ethanol extracts. EFV (efavirenz) is used as a positive control and is screened at different concentrations

2.10.3.5 The anti-HIV activity of fractions from liquid-liquid partitioning of the 50% ethanol extract of the four plant mixture

The anti-HIV activity of the concentrated fractions of the 50% ethanol extract of the four-plant mixture is shown in Figure 2.24. The water and 90% ethanol fractions were inactive at all test concentrations. The ethyl acetate fraction was the only fractions that showed anti-HIV activity. The fraction showed anti-HIV activity of $100.6 \pm 1.2\%$ and $64.8 \pm 3.8\%$ at 5 and 2.5 $\mu\text{g/ml}$ with limited cytotoxicity and was inactive at the low-test concentrations. The 50% ethanol extract of the four-plant mixture will be developed further as is, due to the poor anti-HIV activity portrayed by the fractions of this extract.

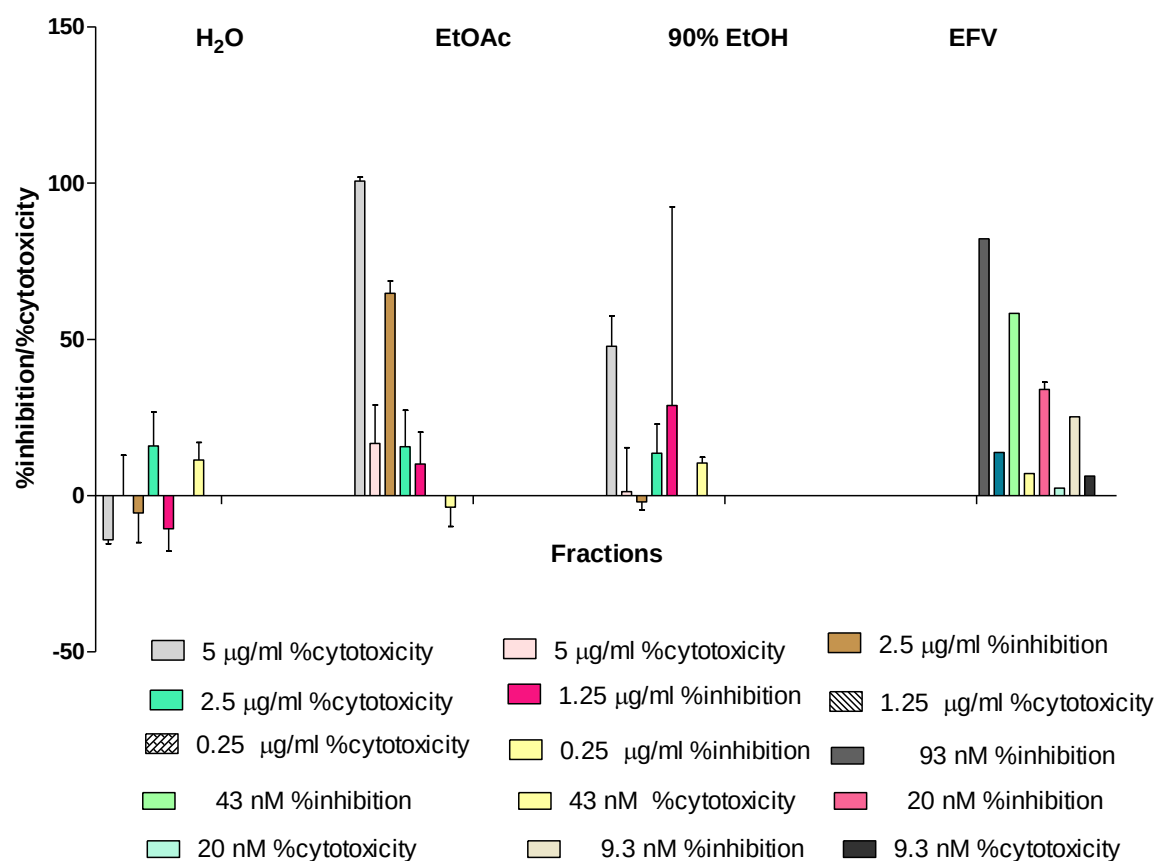


Figure 2.24: Anti-HIV activity of concentrated fractions from the 50% ethanol extract of the four plant mixture through liquid-liquid extraction.

2.10.4 Latency reversal activity of extracts of *G.sericocephala* and the four plant mixture

GFP expression in the cells is an indication of HIV reactivation from latency state.^[54] The levels of GFP expressed by the ethanol, 80% ethanol and the 50% ethanol extracts of *G. sericocephala* is shown in Figure 2.25. The ethanol extract of *G. sericocephala* induced GFP expression by $7.87 \pm 0.74\%$, when screened at $30 \mu\text{g/ml}$ and did not show latency reversal activity at lower test concentrations. The ethanol extract of *G. sericocephala* at higher test concentrations showed latency reversal activity that is similar to the positive control prostratin ($6.97 \pm 1.4\%$). The 80% ethanol of *G. sericocephala* induced GFP expression by $1.19 \pm 0.2\%$ and the 50% ethanol by $3.94 \pm 0.34\%$ at $10 \mu\text{g/ml}$, these two extracts did not exhibit latency reversal activities. The high heterogeneous nature of HIV latent reservoirs makes it highly impossible for a single compound to activate all latent cells. This explains the inability of the 80% ethanol and 50% ethanol extracts to induce GFP expression.

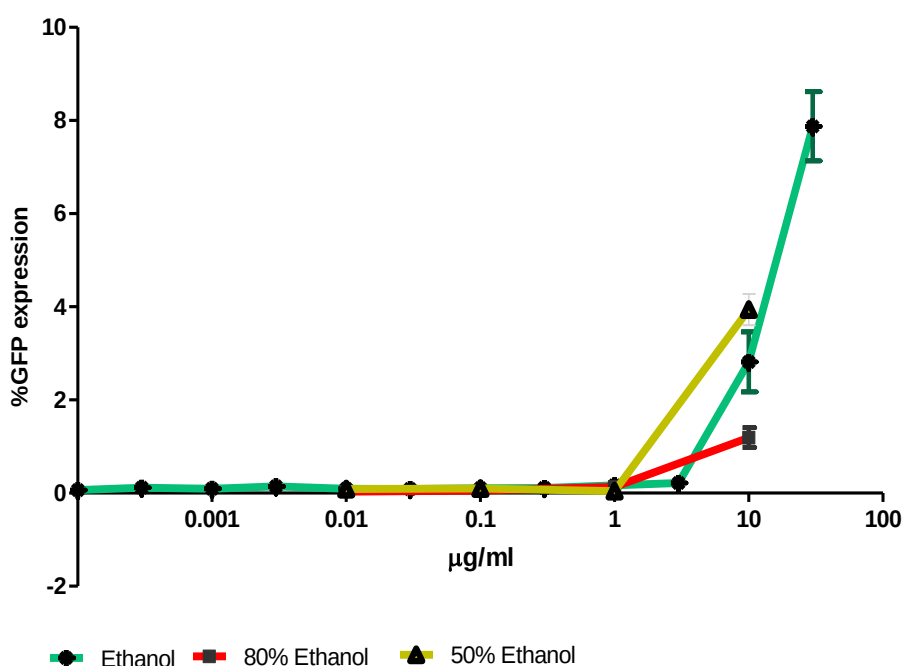


Figure 2.25: GFP expression activity of *G. sericocephala* ethanol 50%, 80% ethanol and ethanol in J-Lat 9.2 cells. Prostratin was used as positive control at $10 \mu\text{M}$ with %GFP expression of $6.965 \pm 1.4\%$ and viability of $0.49 \pm 0.13\%$ (see supplementary data 1).

The latency reversal activity of the ethanol, 80% ethanol and the 50% ethanol extracts of the four plant mixture is shown in Figure 2.26. The 50% ethanol and 80% ethanol extracts induced GFP expression when screened at 10 $\mu\text{g/ml}$ by $14.05\pm0.6\%$ and $13.14\pm0.6\%$, respectively. The 50% ethanol extract of the four plant mixture also induced GFP expression by $11.4\pm0.0\%$ at 1 $\mu\text{g/ml}$. All the extracts did not exhibit latency reversal activity at concentrations lower than 1 $\mu\text{g/ml}$. The 50% ethanol extract showed the most promising GFP expression and was selected for further development due to its good latency reversal activity at 1 $\mu\text{g/ml}$.

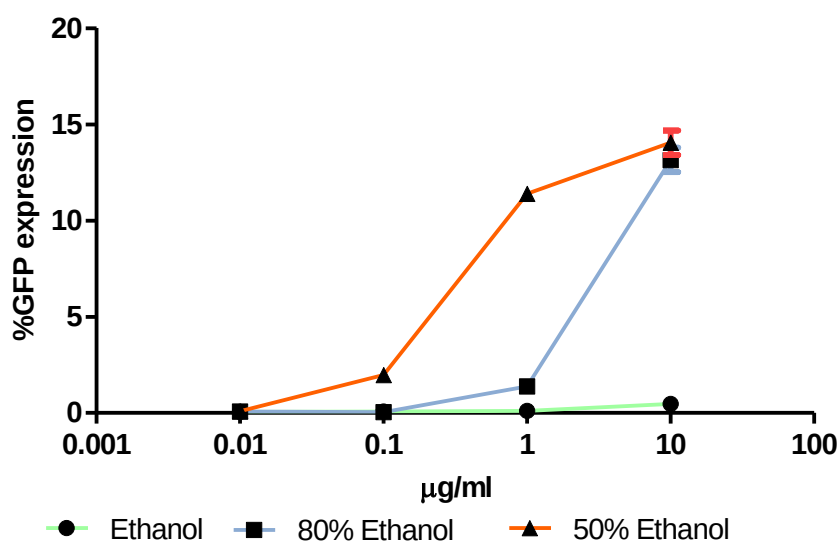


Figure 2.26: GFP expression activity of the 50% ethanol, 80% ethanol and 50% ethanol extracts of the four plant mixture in J-Lat 9.2 cells. Prostratin was used as positive control at 10 μM with %GFP expression of $6.965\pm1.4\%$ and viability of $0.49\pm0.13\%$ (see supplementary data 1)

Different classes of compounds from natural products have been reported to activate the HIV latent reservoirs.^[75] The heterogenous nature of the cells in these reservoirs confirms the inactivity shown by some of the extracts and how the synergistic effect of the four plant mixture could be playing on the activation of these cells. The 50% ethanol extract of the the four plant mixture can also be used as a PKC activator as it showed GFP expressions similar to those of the positive control prostratin.

2.10.4.1 Latency reversal activity of fractions obtained through a separate liquid liquid partitioning of the ethanol extract of *G.sericocephala* and the 50% ethanol extract of the four plant mixture

The latency reversal activity of the fractions from the ethanol extract of *G. sericocephala* and the fractions obtained from the 50% ethanol extract of the four plant mixture is shown in Figure 2.27. The water fraction obtained through liquid-liquid partitioning between ethyl acetate and water of the ethanol extract of *G. sericocephala* did not show any latency reversal activity at all test concentrations. The 90% ethanol fraction obtained through liquid-liquid partitioning between hexane and 90% ethanol of the plant showed good latency reversal activity of greater than 75% at 30 and 10 $\mu\text{g/ml}$ and was inactive at the lower test concentrations. The ethyl acetate fraction induced latency reversal with an IC_{50} of 0.9 $\mu\text{g/ml}$ and was chosen for further development.

The latency reversal activity (GFP expression) of the four plant mixture fractions shows that the water and the 90% ethanol fraction do not exhibit any latency reversal. Consequently, the ethyl acetate fraction of the four plant mixture induced GFP expression with an IC_{50} of 10 $\mu\text{g/ml}$.

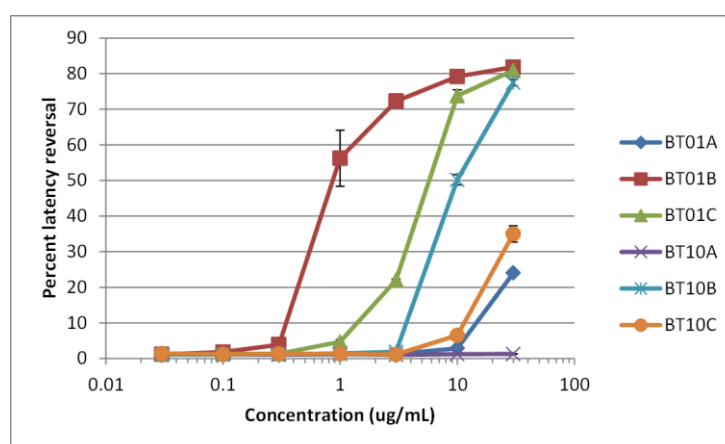


Figure 2.27: Latency reversal activity of concentrated fractions from the ethanol extract of *G. sericocephala* and the 50% ethanol extract of the four plant. BT01A (water), BT01B (ethyl acetate), BT01C (90% ethanol) fractions of the ethanol extract from *G. sericocephala*. BT10A (water), BT10B (ethyl acetate) and BT10C (90% ethanol) fractions of the 50% ethanol extract from the four plant mixture.

The cell viability of all fractions from the four plant mixture and *G.sericocephala* at different test concentrations is shown in Figure 2.28. The ethyl acetate fraction of *G. sericocephala* did not exhibit cell viability at all test concentrations. The water fraction and the 90% ethanol fractions of *G. sericocephala* exhibited cell viability at 30 µg/ml. All fractions from the four plant mixture did not exhibit any cell viability at all test concentrations.

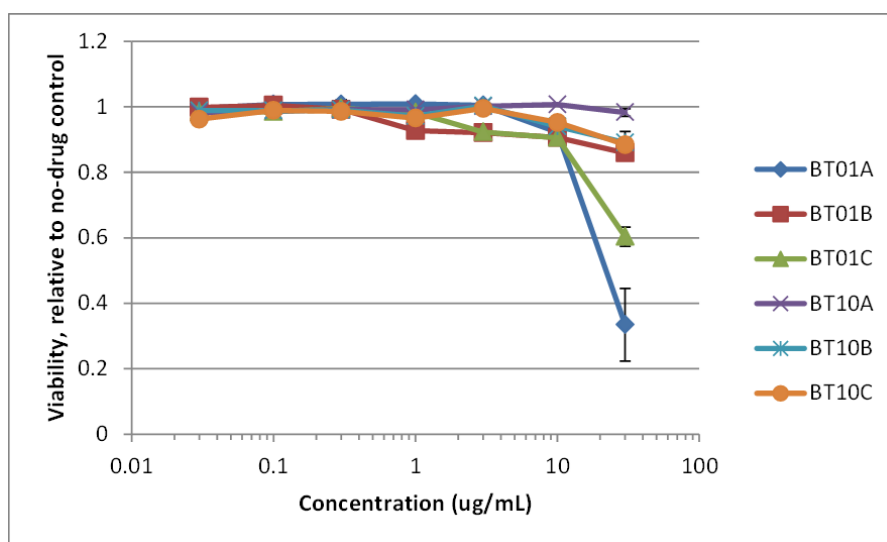


Figure 2.28: Cell viability of concentrated fractions from the ethanol extract of *G. sericocephala* and the 50% ethanol extract of the four plant mixture. BT01A (water), BT01B (ethyl acetate), BT01C (90% ethanol) fractions of the ethanol extract from *G. sericocephala*. BT10A (water), BT10B (ethyl acetate) and BT10C (90% ethanol) fractions of the 50% ethanol fractions from the four plant mixture

The latency reversal activity of the concentrated extracts was only improved for the ethyl acetate fraction of *G. sericocephala*. This fraction showed improved latency reversal activity with no visible cell viability compared to the ethanol extract and will be developed further. The concentrated fractions of the four plant mixture showed poor latency reversal activity compared to the 50% ethanol extract. The 50% ethanol extract of the four plant mixture will be developed further in its form.

2.11 UPLC-QTOF-MS chemical profiling extracts and fractions

2.11.1 Chemical fingerprinting of the DCM extract of *G.sericocephala* roots

The chemical profile of the DCM extract of *G. sericocephala* roots was obtained using UPLC-QTOF-MS Figure 2.29. The positive ESI mode was chosen as it gave better ionization for the identification of compounds. A total of 16 compounds were identified as shown in Table 2.10. The compounds belonging to a variety of different chemical classes such as coumarins, terpenes and neolignans were detected and tentatively identified by generating molecular formulas from MassLynx V 4.1 based on their isotopic fit value (iFit value) and by comparison of MS/MS fragmentation pattern by using online compound databases such as Metlin, Metfusion, ChemSpider, Pubchem Chemical Entities of Biological Interest (CHEBI) and Reaxys.

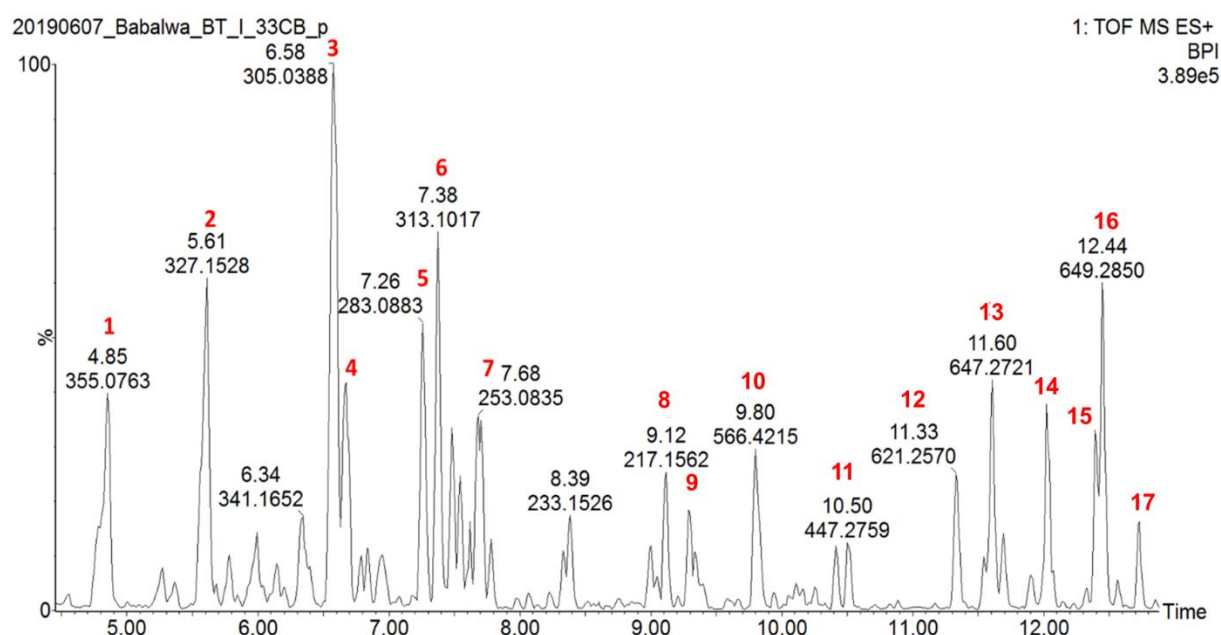


Figure 2. 29: UPLC-QTOF-MS profile of the DCM extract of *Gnidia sericocephala* roots in ESI positive mode BPI chromatogram (0-18 minutes). Compounds detected eluted from 4.85 minutes to 13.13 minutes.

The $[M+H]^+$ observed for peak 1 at m/z 355.0763 at retention time 4.85 minutes corresponded to the molecular formula $C_{19}H_{14}O_7$. The MS/MS spectra revealed the presence of fragment ion at m/z 277 due to a subsequent loss of three water molecules and a retro Diels Alder

rearrangement in its carbonyl moiety. The fragment ion at m/z 221.0560 $[M-C_2H_2]^+$ was due to a McLafferty rearrangement. The fragmentation pattern led to the compound being tentatively identified as 5-methoxysterigmatocystin.^[78] The $[M+H]^+$ ion observed for peak 2 at m/z 327.1412 corresponded to the molecular formula $C_{20}H_{23}O_4$. The MS/MS spectra revealed the presence of a fragment ion at m/z 295.1268 $[M-CH_3O]^+$ suggesting that the molecule had experienced a cleavage on its aldehyde functional group in the aromatic moiety of the molecule, followed by a subsequent loss of the phenol ring to give fragment ion at m/z 202.0756 $[M-C_6H_4OH]^+$. The molecule also experienced a retro Diels-Alder rearrangement to give fragment ion at m/z 137.0589. The compound was tentatively identified as dehydrodiisoeugenol.^[79] The $[M+H]^+$ ion observed at peak 3 at m/z 305.0388 corresponded to a molecular formula of $C_{18}H_9O_5$. The MS/MS spectra revealed the presence of a fragment ions m/z 277.0630 and m/z 249.0557 due to a loss of a carbonyl moiety. The loss of a water molecule was shown by the presence of fragment ion at m/z 221.0598, this led to a tentative identification of the compound being lasioerin.^[78] For peak 4 the $[M+H]^+$ ion was observed at m/z 337.0630 and corresponded to molecular formula $C_{19}H_{13}O_6$. The MS/MS spectra showed the presence of a fragment ion at m/z 321.0350 which represented an exposure to the McLafferty rearrangement and a fragment ion with a formula of $C_{18}H_9O_6$ was produced. The MS/MS spectra of this molecule also showed a subsequent loss of two water molecules followed by a retro-Diels Alder mechanism to give fragment ion at m/z 237.0506. The molecule also experienced the loss of a water molecule to give fragment ion at m/z 221.0549. This molecule was tentatively identified as lasiocephalin.^[78] The $[M+H]^+$ observed for peak 5 at m/z 283.0883 corresponded to a molecular formula of $C_{17}H_{14}O_4$. The MS/MS spectra of the molecule revealed the presence of a fragmentation ion at m/z 267.0579 $[M-CH_3]^+$ due to a methyl loss, followed by a cleavage of a carbonyl group to give fragment ion at m/z 221.0598 $[M-CH_2O_2]^+$. The fragmentation pattern given by this molecule resulted in the molecule being tentatively identified as vadimezan. The $[M+H]^+$ observed for peak 6 at m/z 313.1017 corresponded to molecular formula $C_{18}H_{17}O_5$. The MS/MS spectra of the molecule revealed the presence of fragment ion at m/z 335.0201 $[M-Na]^+$ due to a sodium adduct. The molecule also experienced a McLafferty rearrangement

and gave fragment ion at m/z 237.0552 $[M-C_6H_5]^+$ followed by a retro Diels-Alder rearrangement to give fragment ion at m/z 181.0654 which led to the tentative identification of the molecule being trimethoxyflavone. The $[M+H]^+$ observed for peak 7 at m/z 253.0835 corresponded to molecular formula $C_{16}H_{13}O_3$. The MS/MS spectra revealed the presence of a fragmentation ion at m/z 238.0618 $[M-15]$ due to the loss of a methyl group. The fragment ion at m/z 221.0602 was due to the loss of a methoxy group. A cleavage followed by a loss of an acetyl group was represented by fragment ion at m/z 210.0677. Based on this data the compound was tentatively identified as 3''-methoxyflavone.^[80] Peak 9 $[M+H]^+$ observed at m/z 283.0964 corresponded to a molecular formula of $C_{17}H_{15}O_4$. The MS/MS spectra revealed the presence of fragment ion at m/z 269.0813 $[M-12]$ due to a loss of a methyl and subsequently followed by a loss of a water molecule to give fragment ion at m/z 251.0692. The subsequent loss of a methoxy group revealed by the presence of the fragment ion at m/z 221.0614. The compound was tentatively identified as 7-hydroxy-3-(4-methoxyphenyl)-4-methylcoumarin.^[79] The $[M+H]^+$ ions observed for peaks 8, 10 and 11 at m/z 217.1562, 566.4215 and 447.2759, respectively, were unidentified.

A class of compounds belonging to the daphnane diterpenoids were also tentatively identified in the extract. These compounds were present from peaks 12 to peak 17. The observed $[M+H]^+$ for peak 12 at m/z 621.2570 with chemical formula of $C_{35}H_{41}O_{10}$ was tentatively identified as yuanhuacine A. The $[M+H]^+$ ion observed for peak 13 at m/z 647.2721 with chemical formula of $C_{37}H_{42}O_{10}$ was tentatively identified as yuanhuajine. For peak 14 the $[M+H]^+$ ion observed at m/z 647.2721 with chemical formula of $C_{37}H_{42}O_{10}$ was tentatively identified as gniditrin. Gniditrin has been reported to possess anti-HIV properties as it possesses entry inhibition activities by inhibiting tropic variants of HIV-1 such as CXCR4 and CCR5 variants.^[70,71] The observed $[M+H]^+$ observed for peak 15 and 16 m/z 649.2850 were tentatively identified as gnididin and yuanhuacine, respectively. These two compounds differ in structure by the ester moiety found in the alkyl chain of gnididin and in the benzyl region in yuanhuacine.^[70] Both these compounds have been reported to possess anti-HIV activity.^[70,71]

The $[M+H]^+$ observed for peak 17 at m/z 665.3212 with the chemical formula of $C_{38}H_{49}O_{10}$ was tentatively identified as mezerein.^[72] Mezerein compound is reported to possess anti-HIV properties as well as anti-tumor properties.^[72] The tentative identification of daphnane diterpenoids in the extract demonstrated the success in the technique in being able to identify the possible bioactive compounds, thereby simplifying the targeted purification of the active ingredient.^[39,72,76]

Table 2.11: Compounds tentatively identified in the DCM extract of *G. sericocephala* by UPLC-QTOF-MS

Peak No.	RT (min)	Observed $[M+H]^+$	Molecular Formula	Theoretical $[M+H]^+$	Mass error (ppm)	MS/MS Fragments	Tentative identification
1.	4.85	355.0724	$C_{19}H_{14}O_7$	355.0759		277.0448	5-methoxysterigmato cystin
						249.0485	
						221.0549	
						165.0691	
2.	5.61	327.327.15 28	$C_{20}H_{23}O_4$	327.1412	3.1	231.0753	dehydrodiisoeugenol
						160.9926	
						137.0590	
3.	6.58	305.0388	$C_{18}H_9O_5$	305.2448	3.6	249.0562	lasioerin
						221.0590	
						165.0685	
4	6.69	337.0630	$C_{19}H_{13}O_6$	337.0707	0.3	321.0410	Lasiocephalin
						237.0594	
						133.0303	
5.	7.26	283.0883	$C_{17}H_{14}O_4$	283.0968		267.0579	vadimezan
						221.0598	
						165.0691	
6	7.38	313.1017	$C_{18}H_{17}O_5$	313.1075	1.3	335.0201	trimethoxyflavone
						237.0552	
						181.0654	
						91.0548	
7	7.68	253.0835	$C_{16}H_{13}O_3$	253.0818	1.7	211.0215	anisindione
						183.0291	
						135.0441	
8	9.12	217.1562	$C_{15}H_{21}O$	217.1589		Not detected	unidentified
9	9.68	283.0835	$C_{17}H_{15}O_4$	283.0967	0.6	267.0661	7-hydroxy-3-(4-methoxyphenyl)-4-methylcoumarin
						239.0711	
						165.0713	
						129.0342	

10	9.80	566.4215	Not determined	566.4285		Not detected	unidentified
11	10.50	447.2759	Not determined	447.2885		Not detected	unidentified
12	11.33	621.2570	C ₃₅ H ₄₁ O ₁₀	621.2758	1.8	323.1284	yuanhuacine A
						165.0733	
						123.0819	
13	11.60	647.2721	C ₃₇ H ₄₂ O ₁₀	647.2739	1.6	371.1722	gniditrin
						191.0823	
						165.0708	
14	12.02	647.2721	C ₃₇ H ₄₂ O ₁₀	647.2704	1.6	371.1181	yuanhuajine
						219.0817	
						105.0337	
15	12.39	649.2850	C ₃₇ H ₄₅ O ₁₀	649.0653	0.6	575.4130	gnididin
						503.1699	
						165.0719	
						105.0337	
16	12.44	649.2850	C ₃₇ H ₄₅ O ₁₀	649.2860	1.0	575.4130	yuanhuacine
						503.1699	
						165.0719	
						105.0337	
17	13.13	665.3212	C ₃₈ H ₄₉ O ₁₀	665.3208	0.6	521.2170	mezerein
						385.1835	
						327.2046	
						91.0552	

2.11.2 UPLC-QTOF-MS analysis of *G.sericocephala* extracts obtained using pharmaceutically accepted solvents

The chemical profile of the 50% ethanol, 80% ethanol and the ethanol extracts of *G.sericocephala* were analysed to identify the presence of daphnane diterpenoids in these extracts, obtained using pharmaceutically accepted solvents as shown in Figure 2.25. The presence of these compounds could be used as evidence to support the varying anti-HIV and latency reversal properties seen in the extracts. All extracts showed the presence of yuanhuacine A, yuanhuajine, gniditrin and yuanhuacine. The presence of these compounds support the anti-HIV and latency reversal properties shown by these extracts. The compounds

yuanhuajine and gniditrin in the 50% ethanol extract were visible at low intensities as their peaks were observed to be very close to the baseline. This evidence justified the inactivity of the extract at low test concentrations. In comparison to the 80% ethanol extract, the peak corresponding to yuanhuacine in the ethanol extract was observed as the most intense, moreover, the non-daphnane diterpenoid peaks absent in the ethanol extract that were present in both the 80% ethanol and 50% ethanol extracts could be responsible for the good anti-HIV and latency reversal properties observed in the ethanol at all test concentrations.

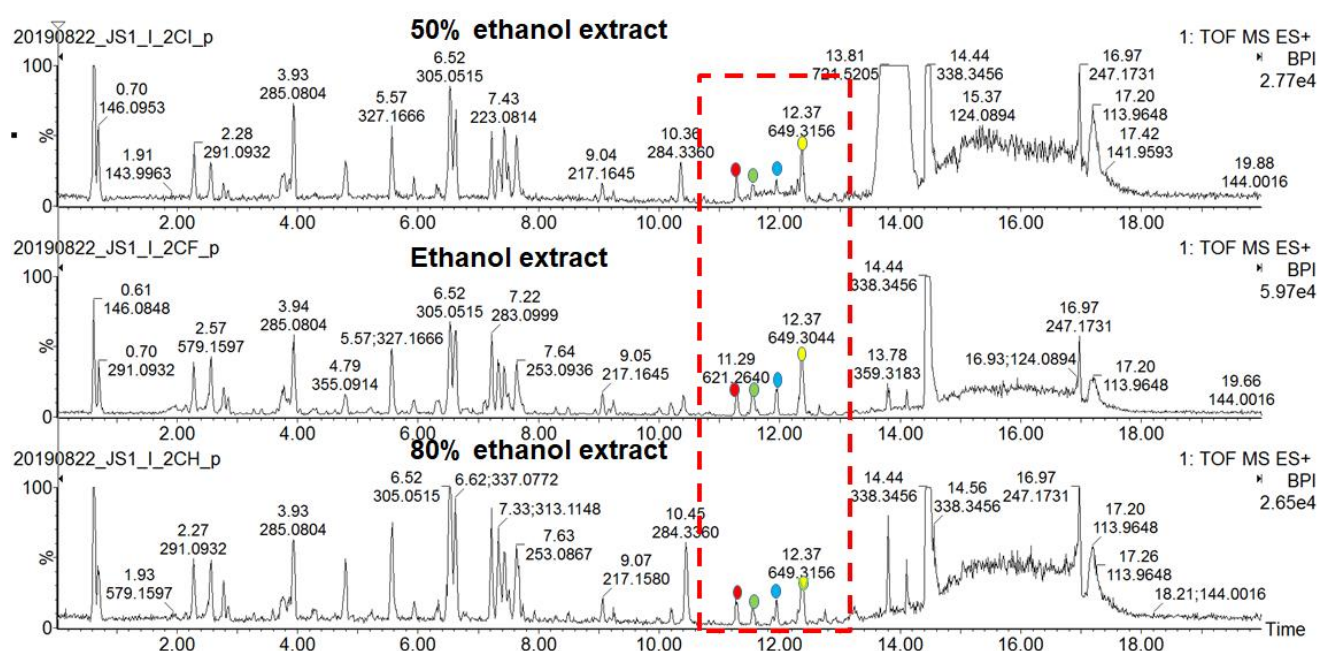


Figure 2. 30: UPLC-QTOF-MS profile of the ethanol, 50% ethanol and 80% ethanol extracts of *G. sericocephala* roots in ESI positive mode BPI chromatogram (0-18 minutes); (red) yuanhuacine A; (green) yuanhuajine; (blue) gniditrin; (yellow) yuanhuacine.

2.11.3 UPLC-QTOF-MS analysis of the ethyl acetate fraction obtained through liquid-liquid partitioning between ethyl acetate and water from the ethanol extract of *G.sericocephala* roots

The chemical analysis of the ethyl acetate fraction was performed to identify peaks that could be responsible for the enhanced anti-HIV and latency reversal activity in the fraction. The chromatogram revealed enhanced daphnane diterpenoids peaks, that were observed to

correspond to peak 1 previously identified as yuanhuacine A, peak 2 (yuanhuajine), peak 3 (gniditrin) and peak 4 (yuanhuacine) as shown in Figure 2.31. Consequently, the chromatogram also revealed low intensity of peaks that were present in the ethanol, which eluted between the 2.61 to 4.82 minute interval region. Based on the retention time of the peaks that were successfully extracted in the ethyl acetate layer, the compounds are medium to non-polar. This showed that the liquid-liquid partitioning between water and ethyl acetate was successful in enhancing the anti-HIV activity.

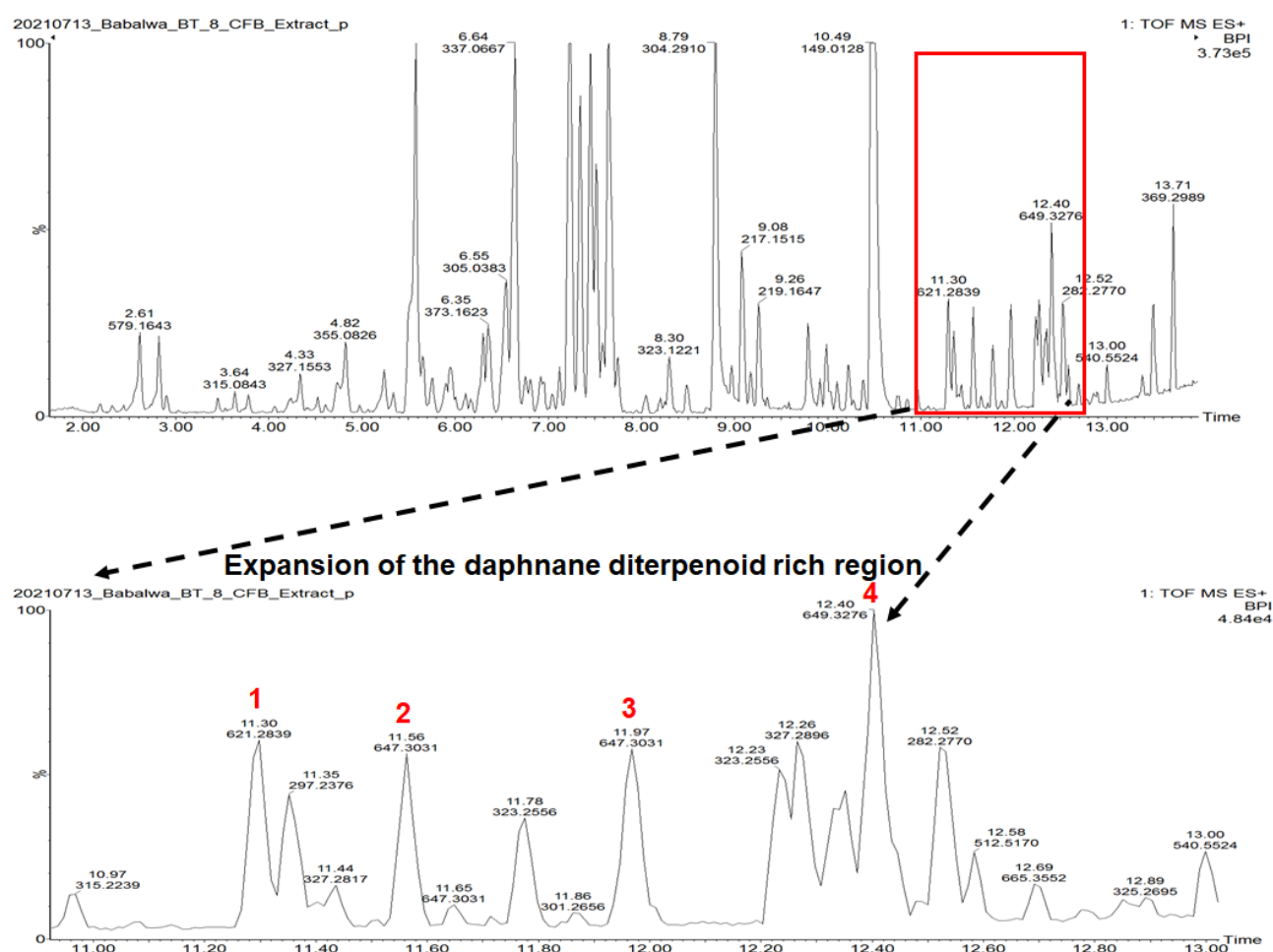


Figure 2. 31: UPLC-QTOF-MS profile of the ethyl acetate fraction of *G. sericocephala* roots in ESI positive mode BPI chromatogram (0-14 minutes). The daphnane diterpenoids were detected between the 11.30 and 12.40 minutes region.

2.11.4 Chemical profile of the 50% ethanol extracts of *S.italica*, *P. pentanisia* and *S. birrea*.

The chemical profile for the *S. italica* roots, *P.prunelloides* roots and the *S. birrea* stems extracted with 50% ethanol was developed for quality control purposes. The compounds were identified and confirmed by processing the ESI negative mode, MS/MS raw data of the extracts using the UNIFI workflow. This was achieved by matching the fragmentation patterns of the compounds with the in build traditional medicine library to provide the chemical structure, molecular formula and the average molecular mass of each compound. For compounds that could not be identified using UNIFI, their identification was done using MassLynx V 4.1 based on their isotopic fit value (iFit value) and by comparison of MS/MS fragmentation pattern using online compound databases such as Metlin, Metfusion, ChemSpider, Pubchem Chemical Entities of Biological Interest (CHEBI) and Reaxys.

2.11.4.1 UPLC-QTOF-MS chemical profiling of the 50% ethanol extract of *S. italica* roots

The chemical profile of the 50% ethanol extract of *S. italica* roots is shown in Figure 2.32. The *S. italica* 50% ethanol extract revealed the presence of 11 major compounds as shown in Table 2.12. A total of eight belonging to the phenolic group which included six anthraquinones, two flavones and one polyphenol as well as one fatty acid and one unidentified compound.

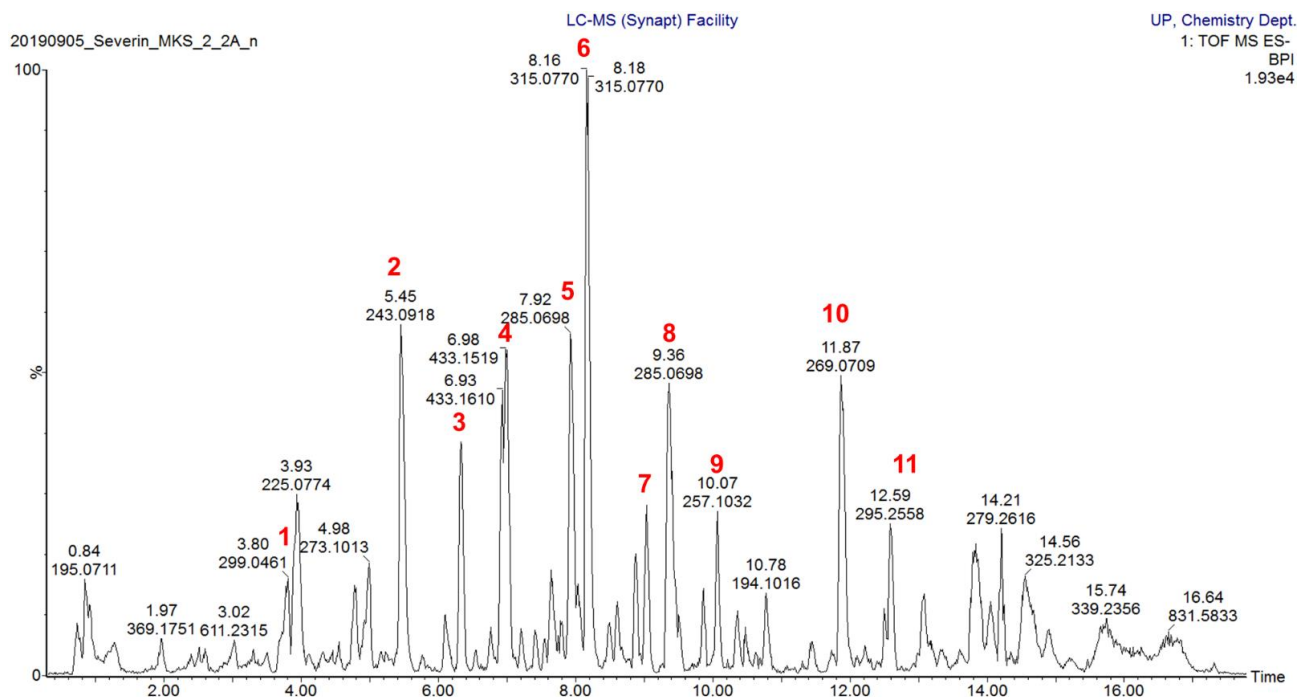


Figure 2.32: UPLC-QTOF-MS profile of the 50% ethanol extract *S. italica* in ESI negative mode BPI chromatogram.

The $[M-H]^-$ ion observed for peak 1 eluting in 3.80 minutes at m/z 299.0461 corresponded to molecular formula $C_{15}H_8O_7$. The compound underwent bombardment and gave fragment ion at m/z 255.0536 due to the loss of a formate ion. The fragment ion at m/z 211.0584 was due to the loss of a CO_2 molecule. Based on the fragmentation patterns the compound was tentatively identified as pseudopurpurin.^[38] The compound is an anthraquinone and is widely reported for its anti-oxidative properties. The $[M-H]^-$ ion observed for peak 2 eluting in 5.45 minutes at m/z 243.0918, the compound was unidentified. The $[M-H]^-$ ion observed for peak 3 eluting in 6.32 minutes at m/z 227.0952 corresponded to molecular formula $C_{14}H_{12}O_3$. The MS/MS spectra revealed the presence of a fragment ion at m/z 143.0483 due to a loss of two water molecules and a retro Diels-Alders rearrangement. This molecule was tentatively identified as resveratrol.^[5] The compound resveratrol is reported as an anti-bacterial compound, which could be playing a part in opportunistic diseases arising due to HIV.^[77] The $[M-H]^-$ ion observed for peak 4 eluting in 6.98 minutes at m/z 433.1519 corresponded to molecular formula $C_{21}H_{22}O_{10}$. The MS/MS spectra revealed the presence of a fragment ion at

m/z 313.0703 due to a loss of $C_4H_8O_4$. The spectra also showed a fragment ion at m/z 270.0517 due to a loss of a sugar moiety. The compound was tentatively identified as 7-hydroxyaloin A.^[10] This compound has been reported for its wound healing properties and has been isolated from other *Senna* species.^[10] The $[M-H]^-$ ion observed for peak 5 eluting in 7.92 minutes at m/z 285.0698 corresponded to molecular formula $C_{15}H_{10}O_6$. The MS/MS spectra revealed the presence of a fragment ion at m/z 269.0790 this resulted from a loss of a water molecule. The spectra also showed a fragment ion at m/z 269.0684 due to a cleavage and a subsequent loss of a carbonyl moiety as well as a McLafferty rearrangement resulting to a loss of an alkene. The compound was tentatively identified as luteolin.^[36] This compound has been isolated and identified in *Senna* species and has been reported to block the function of Tat mediated LTR activity in HIV.^[20] The $[M-H]^-$ ion observed for peak 6 eluting in 8.16 minutes at m/z 315.0485 corresponded to molecular formula $C_{16}H_{12}O_7$. The MS/MS spectra revealed the presence of a fragment ion at m/z 271.0208 this resulted from a loss of the methoxy and a water molecule. The MS/MS spectra also showed a fragment ion at m/z 227.0318 due to cleavage and a loss of an alkene. The compound was tentatively identified as 1,3,5,8-tetrahydroxy-6-methoxy-2-methyl anthraquinone.^[4] The $[M-H]^-$ ion observed for peak 7 eluting in 9.03 minutes at m/z 417.1629 corresponded to molecular formula $C_{21}H_{22}O_9$. The MS/MS spectra revealed the presence of a fragment ion at m/z 254.0567 this resulted from a loss of the of the glucopyranoside substituent. The MS/MS spectra also showed fragment ion at m/z 239.0675 due to a loss of a water molecule. The subsequent loss of CH_2OH after a McLafferty rearrangement yielded fragment ion at m/z 211.0765. The compound was tentatively identified as aloin.^[10] The $[M-H]^-$ ion observed for peak 8 eluting in 9.36 minutes at m/z 285.0698 corresponded to molecular formula $C_{15}H_{10}O_6$. The MS/MS spectra of the molecule revealed fragmentation ion at m/z 268.0341 corresponding to a loss of a water molecule followed by a subsequent loss of the hydroxy moiety shown by fragment ion at m/z 241.0465. The fragment ion shown at m/z 211.0363 was due to a loss of two water molecule. The observed fragmentation patterns shown by this molecule led to it being tentatively identified as 6-hydroxyaloeemodin.^[10] The $[M-H]^-$ ion observed for peak 9 eluting in 10.07 minutes at m/z

257.1032 corresponded to molecular formula $C_{15}H_{14}O_4$. The MS/MS spectra of the molecule revealed a presence of fragment ion at m/z 239.0672 is due to a loss of a water molecule. The fragment ion at m/z 215.0691 was due to a retro Diels Alder rearrangement. The molecule was identified as (R)-prechrysophanol.^[5] The $[M-H]^-$ ion observed for peak 10 eluting in 11.87 minutes at m/z 269.0709 corresponded to molecular formula $C_{15}H_{10}O_5$. The MS/MS spectra showed the presence of fragment ion at m/z 239.0187 due to a loss of two water molecules. The molecule was shown to have undergone a cleavage followed by a loss of CH_4 and gave fragment ion at m/z 226.0279 and was tentatively identified as apigenin. The analogues of apigenin have been reported to contain anti-HIV activity.^[78] For peak 11, the observed $[M-H]^-$ ion at m/z 295.0709 eluting in 12.59 minutes corresponded to molecular formula $C_{18}H_{32}O_3$. The MS/MS spectra of the molecule showed a presence of fragment ion at m/z 225.0058 due to a loss of $C_3H_4O_2$. The molecule was tentatively identified as coronaric acid. The identified compounds in the 50% ethanol extract suggests that the plant could be playing a role of being an immune booster.

Table 2.12: Compounds tentatively identified by UPLC-QTOF-MS in 50% ethanol extract of *Senna italica* roots

Peak	RT (min)	Acquired $[M-H]^-$	Molecular Formula	Theoretical mass $[M-H]^-$	Mass error (ppm)	MS/MS fragments	Possible compound
1	3.80	299.0461	$C_{15}H_8O_7$	299.0309	0.0	255.0536	Pseudopurpurin
						211.0584	
						91.0273	
2	5.45	243.0918	$C_{12}H_{12}O_4Na$	243.0656	4.5	159.0644	Unidentified
						137.0253	
3	6.32	227.0952	$C_{14}H_{12}O_3$	227.0708	-4.8	143.0486	Resveratrol
4	6.98	433.1519	$C_{21}H_{22}O_{10}$	433.1135	-3.7	313.0700	7-hydroxyaloin A
						270.0703	
5	7.92	285.0698	$C_{15}H_{10}O_6$	285.1119	-3.5	239.0685	Luteolin
						133.0277	
6	8.16	315.0770	$C_{16}H_{12}O_7$	315.0505	-2.2	271.0237	1,3,5,8-tetrahydroxy-6-methoxy-2-methyl anthraquinone
						227.0318	
						199.0389	
7	9.03	417.1629	$C_{21}H_{22}O_9$	417.1175	-2.6	239.0675	Aloin
						225.0548	
8	9.36	285.0698	$C_{15}H_{10}O_6$	285.0763	-1.1	211.0380	6-hydroxyaloeemodin
						195.0441	

						183.0430	
9	10.07	257.1032	C ₁₅ H ₁₄ O ₄	257.0798	-6.2	239.0696	R)-prechrysophanol
						229.1451	
						116.9260	
10	11.87	269.0709	C ₁₅ H ₁₀ O ₅	269.0450	3.3	225.0554	Apigenin
						195.0445	
						181.0695	
11	12.59	295.2558	C ₁₈ H ₃₂ O ₃	295.2299	8.5	283.2635	coronaric acid
						183.0108	
						152.9934	

2.11.4.2 UPLC-QTOF-MS chemical profiling of the 50% ethanol extract of *P. prunelloides* roots

The chemical profile of the 50% ethanol extract of *P. prunelloides* roots obtained using UPLC-QTOF-MS is shown in Figure 2.33. A total of seven compounds as shown in Table 2.13 were tentatively identified and belonged to different classes such as iridoid glycosides, flavonoids, fatty acids, and disaccharides.

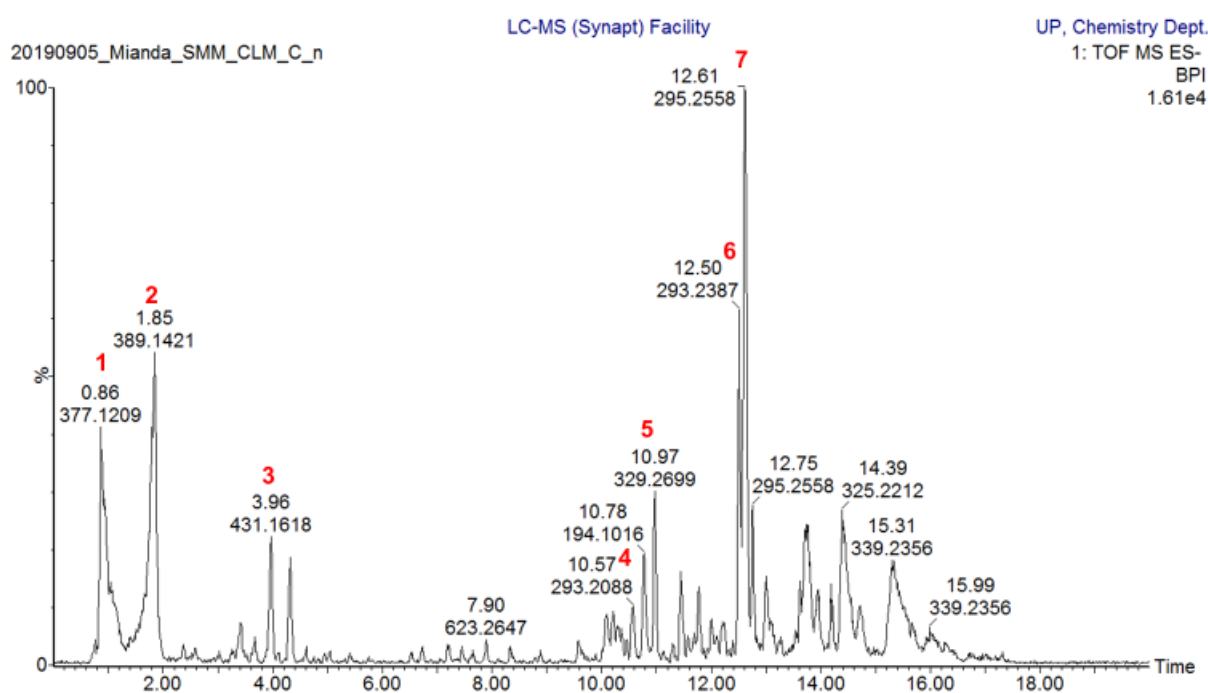


Figure 2.33: UPLC-QTOF-MS profile of the 50% ethanol extract *P. prunelloides* in ESI negative mode BPI chromatogram (0-18 minutes). Compounds identified eluted from 0.86 minutes to 12.61 minutes. Peaks observed after this were also present in the blank (see supplementary data 2).

The UPLC-MS profile of *P. prunelloides* showed an $[M-H]^-$ ion observed for peak 1 at m/z 377.1209 and corresponded to molecular formula $C_{12}H_{22}O_{11}Cl$. The MS/MS spectra showed a fragment ion at m/z 341.1067 due to the loss of the chlorine adduct. The observed fragment ion at m/z 179.0556 was due to a loss of the glycoside bond which resulted to the formation of a fructose and glucose moiety. The compound was tentatively identified as sucrose.^[4] The observed $[M-H]^-$ for peak 2 showed the presence of a precursor ion at m/z 389.1421 corresponding to molecular formula $C_{16}H_{20}O_{11}$. The MS/MS spectra of the peak showed a fragment ion at m/z 227.0574 due to a loss of a glycoside, the fragment ion at m/z 147.0440 was due to a loss of a CH_2O and a H_2O molecule. The compound was identified as monotropein. The $[M-H]^-$ ion observed for peak 3 at m/z 431.1190 corresponded to molecular formula $C_{18}H_{24}O_{12}$. The MS/MS fragment observed at m/z 165.0576 was due to a loss of a glycoside moiety. The compound was tentatively identified as asperulosidic acid.^[35,79] The $[M-H]^-$ ion observed for peak 4 revealed a presence of a precursor ion at m/z 289.0989 corresponding to molecular formula $C_{15}H_{14}O_6$. The MS/MS spectra showed a fragment ion at m/z 245.0851 due to a loss of a flavonoid ring. The fragment ion observed at m/z 137.0238 was due to a retro Diels-Alder rearrangement in its flavonoid ring. The compound was tentatively identified as catechin.^[32] A total of three fatty acids were observed for peaks 5, 6, 7 and were tentatively identified as sanleng, (E,E)-9-oxooctadeca-10,12-dienoic acid and coronaric acid, respectively.^[15]

Table 2.13: Compounds tentatively identified by UPLC-QTOF-MS in 50% ethanol extract of *P. prunelloides*

Peak	RT (min)	Acquired $[M-H]^-$	Molecular Formula	Theoretical mass $[M-H]^-$	Mass error (ppm)	MS/MS fragments	Possible compound
1	0.86	377.1209	$C_{12}H_{22}O_{11}Cl$	377.0846	-1.6	341.1067	sucrose
						179.0556	
						96.5282	
2	1.85	389.1421	$C_{16}H_{20}O_{11}$	389.1063	-5.4	227.0574	monotropein
						147.0440	
						59.0143	
3	3.96	431.1618	$C_{18}H_{24}O_{12}$	431.1190	-4.4	243.0275	asperulosidic acid
						165.0576	
4	4.32	289.0989	$C_{15}H_{14}O_6$	289.0712	-3.8	175.0260	catechin
						159.0396	

						123.0242	
5	10.97	329.2699	C ₁₈ H ₃₄ O ₅	329.2328	9.4	239.0557	sanleng acid
						193.0849	
						125.0987	
6	12.50	293.2387	C ₁₈ H ₃₀ O ₃	293.2107	-3.4	239.0323	(E,E)-9-oxooctadeca-10,12-dienoic acid
						183.0098	
						152.9936	
7	12.61	295.2558	C ₁₈ H ₃₂ O ₃	295.2289	5.4	277.2222	coronaric acid
						269.0490	
						171.1041	

2.11.4.3 UPLC-QTOF-MS chemical profiling of the 50% ethanol extract of *S. birrea* stem bark

The chemical profile of the 50% ethanol extract of *S. birrea* stems showed a total of five compounds as shown in Figure 2.34 and Table 2.14 . The compounds belonged to different classes such as organic acids and flavonoids. The identified compounds are in the region of 0-4 minutes, based on the retention time these compounds are polar. The compounds in the region 4.2 to 18 minutes were regarded as artifacts.

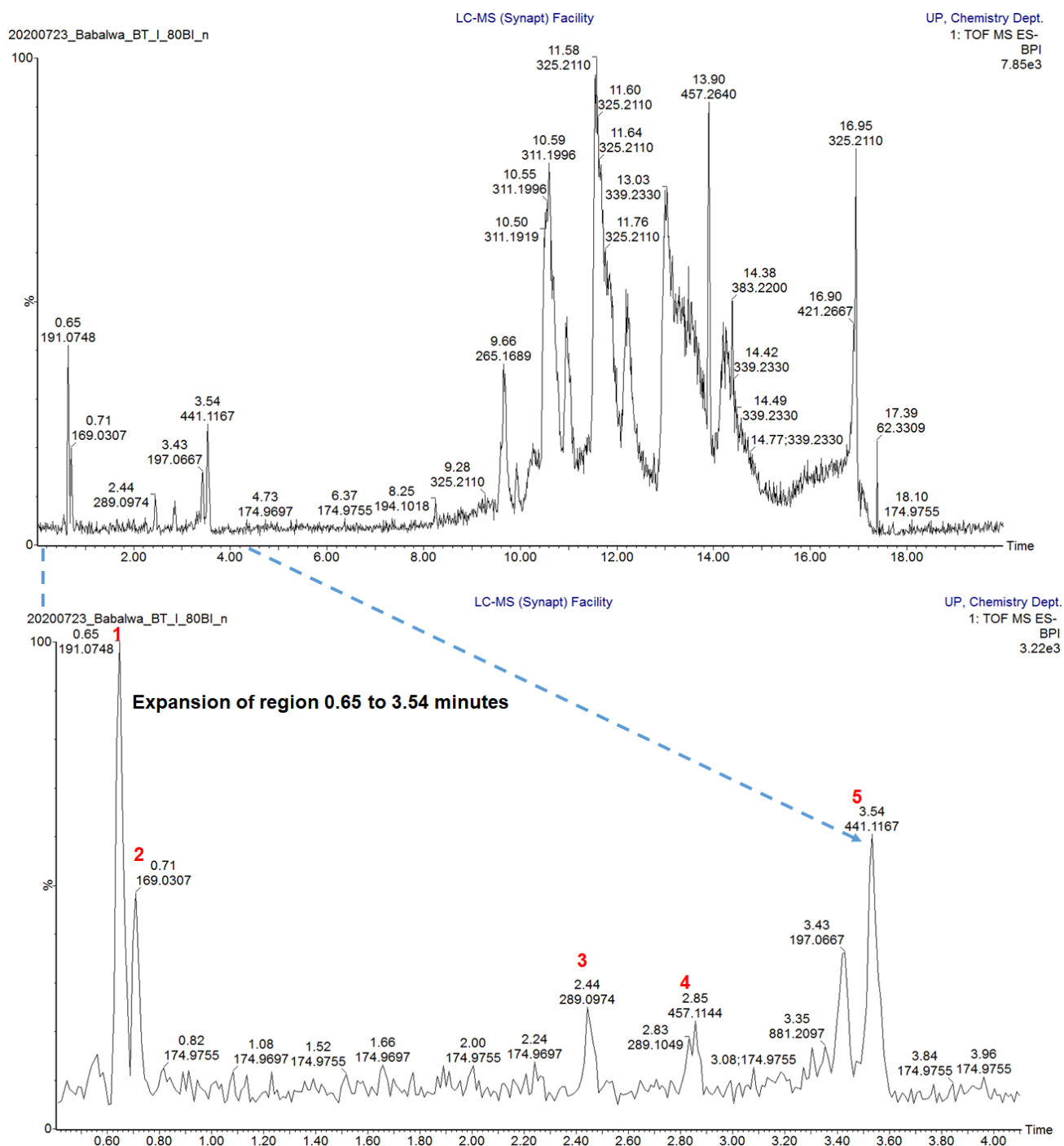


Figure 2.34: UPLC-QTOF-MS profile of the 50% ethanol extract *S. birrea* in ESI negative mode BPI chromatogram (0-18 minutes). Compounds identified eluted from 0.65 minutes to 3.54 minutes. Peaks observed after this retention time were artifacts.

The chromatogram revealed an $[M-H]^-$ ion observed for peak 1 at m/z 191.0748 at retention time of 0.65 minutes. The peak corresponded to molecular formula $C_7H_{12}O_6$. The MS/MS spectra showed fragmentation ion at m/z 85.0351 due to a cleavage of a CO_2 and the

subsequent loss of H₂O and C₂H₆O₂ molecules. This molecule was tentatively identified as quinic acid.^[65] The compound has been reported for its anti-HIV and anti-oxidative properties and was previously identified in the ethanol extract of *S. birrea*.^[77] The [M-H]⁻ ion observed for peak 2 at *m/z* 169.0307 at retention time of 0.71 minutes corresponded to molecular formula C₇H₆O₅. The MS/MS spectra revealed a fragment ion at *m/z* 125.0295 due to a loss of the carboxyl group. The compound was tentatively identified as gallic acid.^[4,31,35] The compound has been reported for its anti-HIV properties and has been reported to be present in the stem bark of *S. birrea*. The [M-H]⁻ ion observed for peak 3 at *m/z* 289.0974 retention time of 2.44 minutes corresponded to molecular formula C₁₅H₁₃O₆. The MS/MS spectra showed a fragment ion at *m/z* 255.0275 due to a loss of two water molecules. There was a subsequent loss of two other water molecules resulting to fragment ion at *m/z* 255.0275. The fragment ion at *m/z* 109.0260 was due to a loss of two water molecules from the parent ion and a loss of C₆H₅O₂ molecule. The compound was tentatively identified as catechin.^[32,80] The [M-H]⁻ ion observed for peak 4 at *m/z* 457.1144 with retention time 2.85 minutes corresponded to molecular formula C₂₂H₁₈O₁₁. The MS/MS spectra showed a fragment ion at *m/z* 301.0522 due to a loss of two water molecules and C₆H₆O₃. The fragment ion at *m/z* 169.0193 was due to a loss of C₇H₆O₅ molecule. The molecule was tentatively identified as epigallocatechin gallate.^[31,80] The [M-H]⁻ ion observed for peak 5 at *m/z* 441.1167 at retention time of 3.54 minutes corresponding to molecular formula C₁₅H₂₂O₁₅. The MS/MS spectra showed fragmentation ion at *m/z* 169.0203 due to a loss of C₁₅H₁₂O₅. The spectra also showed a fragment ion at *m/z* 125.0288 due to a loss of C₁₅H₁₂O₅ and a loss of CO₂. The molecule was tentatively identified as epicatechin gallate.^[31,80]

Table 2.14: Compounds tentatively identified by UPLC-QTOF-MS in 50% ethanol extract of *S. birrea*.

Peak	RT (min)	Acquired [M-H] ⁺	Molecular Formula	Theoretical mass [M-H] ⁺	Mass error (ppm)	MS/MS fragments	Possible compound
1	0.65	191.0748	C ₇ H ₁₂ O ₆	191.0612	9.5	85.0351	quinic acid
2	0.71	169.0307	C ₇ H ₆ O ₅	169.0137	9.5	125.0295	gallic acid
3	2.44	289.0974	C ₁₅ H ₁₃ O ₆	289.0776	9.5	174.9604 69.0007	catechin
4	2.85	457.1144	C ₂₂ H ₁₇ O ₁₁	457.1136	-1.5	311.066 169.0207 125.0297	epigallocatechin gallate
5	3.54	441.1167	C ₁₅ H ₂₂ O ₁₅	441.0890	2.3	245.0464 169.0203 125.0288	epicatechin gallate
6	9.66	265.1689	C ₁₉ H ₂₂ O	265.1542	-1.0	183.0185 96.6579	1-(penta-methylphenyl)-2-phenylethanone
7	10.59	311.1996	C ₁₃ H ₂₈ O ₈	311.1753	--	183.0187 117.0563	hexanedioic acid
8	11.58	325.2110	C ₁₄ H ₃₀ O ₈	325.1862	-1.0	183.0192 119.0567	hexaoxaecosane-1,20-diol
9	12.19	325.2110	C ₁₄ H ₃₀ O ₈	325.1862	0.5	183.0190 119.0567	hepta-ethylene Glycol

The chemical profile of the 50% ethanol extracts of *S. birrea*, *S. italica* and *P. prunelloides* revealed the presence of compounds which have been vastly reported for their anti-microbial, anti-fungal, anti-oxidative and some with anti-viral activities suggesting that the mode of action for each separate plant could be in the alleviating of opportunistic diseases by boosting the immune system.

2.12 Conclusion

The dichloromethane: methanol extracts of *Senna italica*, *Pentanisia prunelloides*, and *Sclerocarya birrea* stem did not inhibit HIV at 25 and 2.5 µg/ml. The extract from *S. birrea* leaf inhibited the virus at 25 µg/ml only. From this data and the assay conducted in this study, the *S. birrea* leaf contain compounds with anti-HIV activity when compared to the traditional health practitioners *S. birrea* stem. The DCM : MeOH extract of *Gnidia sericocephala* showed very good inhibition activities at 25 and 2.5 µg/ml with minimum cytotoxicity levels. The DCM : MeOH extract of the mixture exhibited good anti-HIV activity mostly at 25 µg/ml. The anti-HIV

activity of the mixture was attributed to the presence of the *G. sericocephala* and the synergistic effect of the compounds present in the four plants extracts. Further screening of the dichloromethane: methanol extract of the mixture and *G. sericocephala* showed significant anti-HIV activity that is dose dependant. *G. sericocephala* sequential extracts were further tested for their anti-HIV activity. The dichloromethane extract of *G. sericocephala* gave the best anti-HIV activity as it was the only extract to exhibit good anti-HIV activity in lower doses. The Hexane extract only showed anti-HIV properties at some concentration and did not exhibit any HIV properties at lower concentrations. The methanol and the ethyl acetate extract only exhibited good anti-HIV properties at higher doses, thus, we will not be going further with them in determining the compounds responsible for the activity. The dichloromethane extract will be investigated further for its anti-HIV properties to determine compounds responsible for the activity. The proven anti-HIV activity of *G. sericocephala* is reported for the first time in this study. These results can be used to support the claims made by the traditional health practitioner. The ethanol extract of *G. sericocephala* and 50% ethanol of the four-plant mixture gave the best anti-HIV activity. The increased anti-HIV activity with minimal cytotoxicity in *G. sericocephala* was achieved after fractionation through liquid-liquid partitioning. The ethyl acetate fraction showed excellent anti-HIV activity as well as latency reversal activity. The 50% ethanol extract of the four-plant mixture gave the best anti-HIV and latency reversal activity. The chemical profiles of the 50% ethanol extracts from *S. birrea*, *S. italica*, *G. sericocephala* and *P. prunelloides* will be used for quality control. The UPLC-MS profile of the ethyl acetate fraction compared to the ethanol extract of *G. sericocephala* showed a decrease in peak intensity of the inactive compounds. The dichloromethane extract was further analysed using UPLC-QTOF-MS to identify compounds that exhibit HIV inhibition activities. The presence of daphnane diterpenoids in the extracts of *G. sericocephala* supported the observed anti-HIV properties possessed by this plant.

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Chapter three

HPLC-based purification and identification of anti-HIV compounds exhibiting latency reversal activity from the dichloromethane root extract and the ethyl acetate fraction of *Gnidia sericocephala*

3.1 Introduction

Plant extracts are composed of complex mixtures of compounds that require further separation and purification to obtain bioactive fractions and single pure active compounds.^[1] Usually, plants biosynthesize these in minor quantities, making it lab intensive and time consuming to get them in their pure form.^[2,3] On the other hand, chromatographic techniques such as column chromatography are used in the initial stages of purification to obtain fractions that contain compounds with similar affinity.^[4] Active fractions are either purified using liquid chromatography, size-exclusion gel filtration, flash chromatography or preparative thin layer chromatography to get the active pure compounds.^[5]

A faster approach to reaching a bio-active compound involves incorporating biological screening to the analytical techniques to achieve bio-selectivity and focus on relevant compounds.^[3] Only compounds with biological binding sites are of interest.^[6] The purification of natural products can involve numerous purification steps to obtain pure compounds where each fraction obtained has to be submitted for bio-assays in order to follow the activity.^[7] Natural products drug discovery can also be accelerated with the use of a protocol that uses HPLC-coupled spectroscopy/spectrometry techniques to allow for the acquisition of structural information on compounds contained in an extract or fraction in a short time (localised activity in extracts).^[7] In this technique, detectors such as ultraviolet (UV), evaporative light scattering detector (ELSD), MS, and sometimes NMR can be incorporated as shown in Figure 3.1. Microfractions are usually collected in a time-based collection in micro titer plates, with wells corresponding to each minute.^[3,8] This method accelerates the localization of activity by correlating discrete HPLC peaks with bioactive data prior preparative scale purification.^[9]

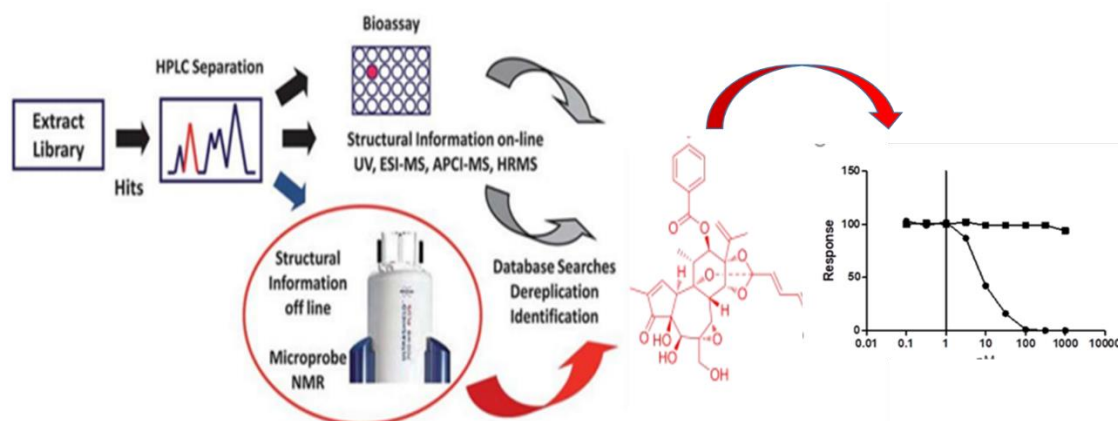


Figure 3. 1: Process for HPLC-based activity profiling in natural product drug discovery, adapted with modifications from Kun Du (2015).^[3]

3.2. Bio-assay guided purification using column chromatography

Column chromatography is one of the oldest techniques in the purification of natural products.^[10] This usually involves packing a column chromatography with silica gel as a stationary phase and organic solvents as mobile phase.^[11] Choosing the correct mobile phase is a factor that needs to be taken into consideration as the compounds need to dissolve in the solvent.^[12] In this technique compounds are separated based on affinity to the stationary phase and the mobile phase.^[13] Collected fractions are combined based on similarity using TLC visualization under ultra-violet light (UV light) or by staining with a reagent.^[11] There are different reagents used for different classes of compounds and they produce various colors corresponding to a group of compounds.^[14] This is the most crucial step in detecting the compounds as one needs to find the best visualizing reagents for compounds.^[15] The setback with this technique involves the difficulty to reproduce the same results, as the technique is a qualitative and not quantitative, analysis is not automated, separation process is limited due to the short lengths of the plate.^[15] On the other hand, size exclusion gel filtration chromatography is an easy to use technique as it only separates compounds based on size of the molecules in solution.^[10] The larger molecules in this technique elute first and this is mostly used in the final purification steps in order to remove impurities in the sample.^[3,16,17]

3.3. High performance liquid chromatography (HPLC) guided purification of natural products

Plant extracts are made up of complex mixtures of compounds^[18]. Hence, it is time-consuming to reach the single pure bio-active compound.^[18] The introduction of bio-assay guided purification provided some relief as it eliminates the purification of inactive compounds. However, it still requires a long time to get to a single pure compound.^[4,12,19] Purification using high performance liquid chromatography on the other hand provides an essential component in the purification of these compounds.^[20] The technique allows for on-line detection of the isolated compounds with real time feedback to sample collectors.^[3,8] The real-time analysis of the instrument allows for the collection of individual fractions without characterization as this part is achieved by using either an evaporative light-scattering detector, ultra-violet light for compounds with a chromophore, and mass spectrometry for ionizing compounds resulting in high purity of compounds for biological screening and structure elucidation using nuclear magnetic resonance spectroscopy.^[3,8]

3.4. HPLC-MS-SPE-NMR is natural products isolation

The identification of compounds of interest at an early stage of natural products drug discovery is one of the crucial steps in the isolation of known or novel bioactive compounds.^[12] On the other hand, compound dereplication methods are widely used for the rapid identification and isolation of compounds. Newer techniques include the integration of HPLC with detectors such as MS and NMR.^[12] Both instruments, the MS and the NMR have proven to be reliable techniques, however, as stand-alone they have shown some limitations.^[12]

MS is an ideal technique in compound identification due to its high sensitivity and ability to detect compounds at nanomolar concentrations.^[13] The application of the tandem mass spectrometry (MS/MS) and online compound library databases have enabled the identification of many compounds with relatively high confidence through spectral matching with authentic compounds in these numerous online data bases.^[12,13] However, accurate

confirmation is often dependent on the ionization efficiency of the molecule and the elimination of interfering ions. Hence, the technique is often coupled to an LC or GC for relative purification prior compound detection to minimize ion interferences and spectral overlap.

NMR offers relative structural elucidation of compounds by identifying nuclei connections within a molecule based on their different electronic environments and magnetic properties with probability of reproducibility as well as quantitative accuracy.^[14] The technique is non-destructive and allows for data collection of molecules with diverse functionalities.^[15] However, NMR still has lower sensitivity and requires a substantial amount of compound for proper identification of compounds.^[12,14] The concentrations of these compounds in plants are typically very low and requires purification and concentration to acquire sufficient amounts to facilitate quality NMR data collection.^[12,13]

Individual compounds from complex crude extracts from plants are rapidly purified and isolated using the hyphenated high performance liquid chromatography–mass spectrometry–solid-phase extraction nuclear magnetic resonance (HPLC-MS-SPE-NMR).^[13,14] Mixtures of compounds are separated in the HPLC columns after which they split into two streams using an adjustable high pressure stream splitter, whereby, 95% of the sample goes to a diode array detector (DAD) or (UV-VIS) detector and 5% to the MS allowing the MS detector to be operated at the higher ionization efficiency. After the DAD detector, water is added to the sample stream with a make-up pump, to decrease the organic solvent proportions of the elution and to promote better adsorption of the peaks on the SPE cartridges.^[15] The multiple trapping of the compounds on the same cartridges is carried out with repeated sample injection on to the HPLC column and trapping a particular peak of interest in order to obtain sufficient amounts prior NMR analysis. All the trapped compounds in SPE cartridges are eluted and transferred into vials and dried for offline NMR analysis or eluted using deuterated solvents for online NMR analysis.^[12,15] Based on this, the hyphenation of HPLC-MS-SPE-NMR system offers a comprehensive information for plant extracts including hydrophobicity,

UV-visible absorption, atomic connectivity (from NMR), resulting in the rapid and effective structural elucidation of compounds.^[15]

3.5. Daphnane diterpenoids as drug candidates in HIV treatment

Daphnane diterpenoids are natural products compounds mainly distributed in the *Thymelaeaceae* and *Euphorbiaceae* families possess various pharmacological properties and are highly toxic.^[20] However, plants biosynthesise them in small quantities. The compounds are typically found in plants as 5/7/6 tricyclic ring systems with an orthoester motif and sometimes a macrocyclic ring. According to Jin *et al.*, (2012), these are highly functionalised, highly oxygenated with numerous stereocenters with the orthoester motif formed between carbons 9,12,13 or 9,13,14 and 12,13,14 and are highly oxygenated hydroxyl group formed in its macrocyclic ring. ^[21] These functionalities are reported to be crucial for the anti-HIV activity.^[20] The anti-HIV activity portrayed by daphnane diterpenoids is mostly seen from daphnane diterpenoids with the orthoester moiety at C-9, C-13 and C-14.^[23]

In this study the daphnane diterpenoids were purified by following a HPLC guided fractionation in order to localize the bioactive compounds in the extracts. The HPLC-MS-SPE and compound dereplication using a mass directed HPLC purification were the techniques used for rapid identification of compounds. However, the HPLC microfractionation technique carried out at an analytical scale. Hence, the use of column chromatography was used to reproduce the active fractions at a preparative scale. This incorporation of multiple purification techniques proved to be an ideal purification strategy for the purification of daphnane diterpenoids targeted in this study.

3.6. Methods

3.6.1. Time based activity localization of the DCM extract using HPLC

The activity localization of the extract was performed on a Waters chromatographic system (Waters 600 controller). The sample was prepared by dissolving 10 mg of extract in 1 ml of a Waters ROMIL HPLC grade acetonitrile to obtain a concentration of 10 mg/ml. The particulate matter was removed by centrifuging (2000 rpm) the solution for 10 minutes and filtered through a 0.22 μm (Millipore) membrane syringe filter unit. A Phenomex, Luna 5 μ reverse phase column with particle size 250 x 4.60 mm, 5 microns was used as a stationary phase. The mobile phase consisted of water in 0.05% trifluoroacetic acid (solvent A) and acetonitrile in 0.05% trifluoroacetic acid (solvent B). A gradient elution of 5-95% solvent B was carried out for 30 min at a flow rate of 1ml/min. An injection volume of 30 μl corresponding to 300 μg of sample was used. Data was collected using Millenium software version (Waters). This was repeated three times and the deep-well plates were evaporated to dryness on a Genevac EZ-2, one deep-well plates was kept for biological anti-HIV screening, the second one for UPLC-QTOF-MS analysis and the last plate was kept as reference as shown in Figure 3.2.

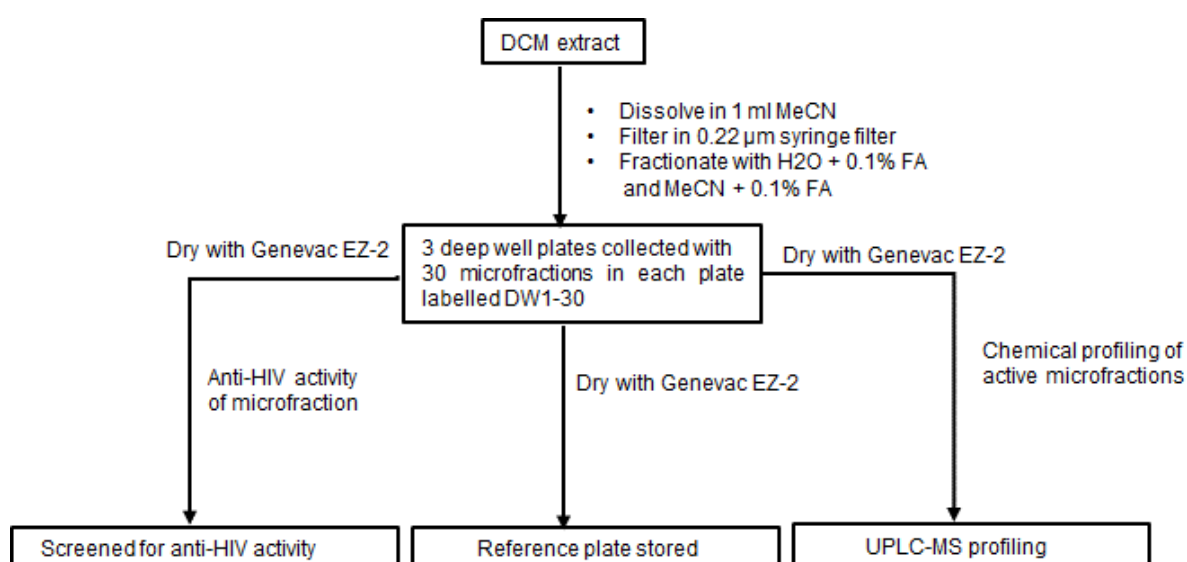


Figure 3. 2: Process for the activity localization of the DCM extract

3.6.2. Fractionation using silica gel column chromatography

The DCM extract (16 g) was separated using an open column chromatography of 107 length and 7 cm diameter on silica gel (0.063- 0.2 mm). The column was packed using a slurry suspension formed by mixing 1.5 kg of dried silica gel (0.063-0.2 mm/70-230 mesh) with DCM: n-hexane (1:1). The extract was dissolved in 5 ml DCM for loading, following a stepwise gradient of DCM : n-hex A; acetone B; 100% A (1 L), 5% B (1 L), 10% B (1L), 10% B (1 L), 15% B (1.5 L), 20% B (1.5 L), 30% B (2 L), 50% B (2 L), 100% B (2 L) and final wash with acetone : methanol (80:20) (3 L) at a flow rate of 5 ml/min. The obtained fractions were further analysed by TLC using silica gel plates (60 F₂₅₄) and visualised under UV light at 254 nm followed by staining with vanillin. Similar fractions were combined based on their R_f values. After combining the similar fractions analysed using UPLC-QTOF-MS. The active fractions were further purified using preparative HPLC-MS.

3.6.3. Fractionation using flash chromatography

The fractionation of the DCM extract using flash chromatography was carried out on a BUCHI Pure C-815-Flash system. Due to insufficient quantities of the DCM extract, the similar chemical profile between the DCM extract and the ethyl acetate fraction of *G. sericocephala*, obtained from the pilot scale extraction (See Chapter , section 4.4.2.1) was thereby used for further purification of yuanhuacine, and the cis 2"/3" isomer of gnididin.

The DCM extract (20 g) and the ethyl acetate fraction (30 g) were separately dissolved in 30 ml of DCM and 30 ml ethyl acetate, respectively. The particulate matter was removed by centrifuging (2000 rpm) the solution for 10 min and filtered through a 0.22 µm (Millipore) membrane syringe filter unit. The filtered DCM extract was injected into a 220 g FlashPureEcoFlex silica (50 µm irregular) cartridge and eluted using hexane A: acetone B; 15% B (5 min), 20% B (20 min), 30% B (20 min), 50% B (10 min), 100% B (20 min) at a low rate of 120 ml/min in 80 minutes. The compounds were detected using an evaporative light

scattering detector and UV at wavelengths (215, 230, 254 and 279 nm). The fractions obtained from the DCM extract were combined based on TLC and chemical analysis using UPLC-QTOF-MS to identify compounds in the active fractions. The same was followed for the fractionation of the ethyl acetate fraction. Further purification was done using HPLC-MS.

3.6.4. HPLC time-based fractionation of the major compound in *G. sericocephala* roots

The major compounds in the DCM extract (4.38g) of *G. sericocephala* roots were purified using a semi-preparative HPLC on a Waters chromatographic system (600 Controller). A Phenomex, Luna 10 µm reverse phase C₁₈ column with particle size 250 x 10 mm, 5 microns was used as a stationary phase. The mobile phase consisted of water in 0.05% trifluoro acetic acid (solvent A) and acetonitrile in 0.05% trifluoro acetic acid (solvent B). An isocratic method consisting of (50:50) solvent A and solvent B was carried out for 30 min at a flow rate of 3 ml/min. An injection volume of 50 µl corresponding to 1.5 mg of sample per injection. The UV detector was set at wavelength 254 nm and 279 nm for detection. Data was collected on Millenium software (Waters). A time-based collection was used for the collection of the major peak consisting of two inseparable compounds based on UV.

3.6.5. HPLC-MS-SPE purification of the major compounds

The HPLC-MS-SPE purification of the major compounds was performed using an Agilent 1260 Infinity HPLC with a PDA detector coupled to a Bruker AmaZon SL ion trap MS, Bruker Prospect II SPE Interface, and a Bruker Sample Pro sample handler. The inseparable compounds (3 mg) obtained from the HPLC time-based fractionation were resuspended in 1 ml acetonitrile. A repeated injection of 20 µl the solution of the sample was injected on a Phenomenex Luna 5 µm polar C18 (150 × 4.6 mm) column (Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in CH₃OH (C) and 0.1% formic acid in H₂O (D). The following method was applied with an HPLC flow rate of 0.5 mL/ min: linear gradient from 0 to 14 min (40–100% C), 14–25 min (100% C), 25–26 (100–40% C), and 26–30 min (40% C).

Additional water (D) was continuously added at a flow rate of 1.5 mL/min using the makeup Knauer pump to facilitate trapping. The two peaks eluting from the HPLC were repeatedly trapped on Spark Holland GP SPE cartridges using the multitraping function to obtain compound 1 and compound 2 as shown in Figure 3.3.

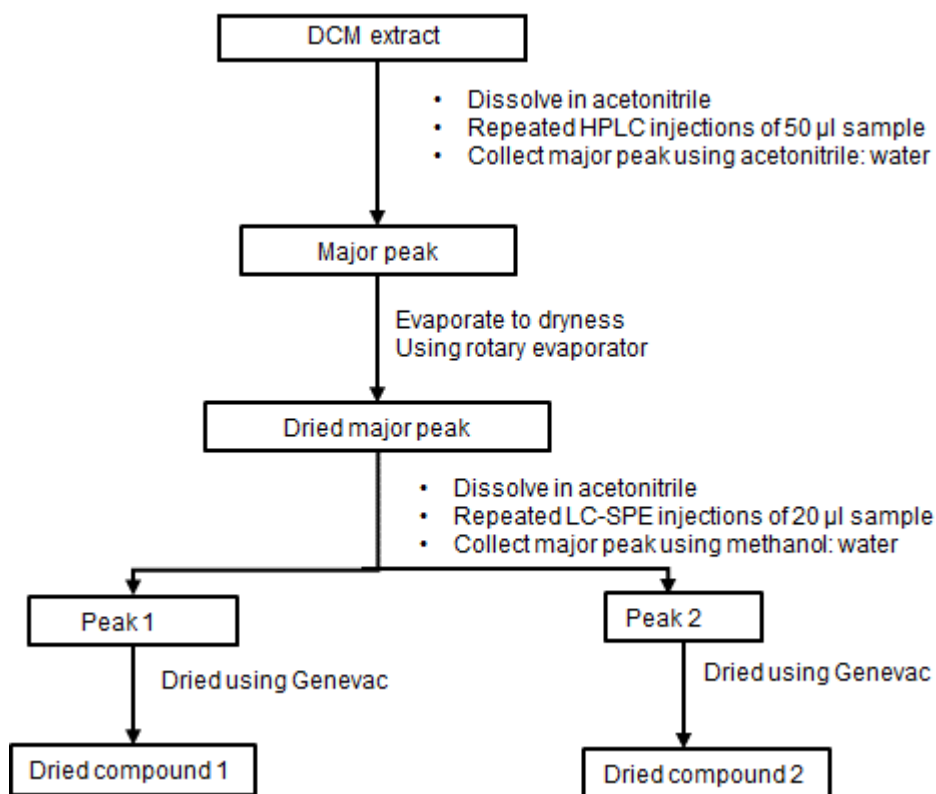


Figure 3.3: Purification of the major peak in the DCM extract using HPLC with a UV detector and final purification using LC-SPE to obtain two compounds.

3.6.6. High performance liquid chromatography-mass spectrometry purification of the active compounds from the DCM extract and the ethyl acetate fraction

The active fractions obtained from the DCM extract using column and flash chromatography were purified using a preparative HPLC-MS on a Waters chromatographic system coupled to a Waters PDA (2998) and interfaced with an Acquity QDa mass spectrometer (Waters, USA). Each of the active fractions (100 mg) was dissolved in 1 ml tetrahydrofuran. The separation of the fractions was performed on an X-Bridge preparative C18 column (19×250 mm, id., 5 µm

particle size, Waters). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 20 ml/min. Gradient elution was applied as follows: 95% A: 5% B for 1 min, 50% A: 50% B (1:00-8:00 min), 15% A: 85% B (8:00-13:00 min), 95% A: 5% B (13:00-13:20 min). Injection volume was 150 μ l.

The same system mentioned above was used for the purification of the fractions obtained from the ethyl acetate fraction. A two-step chromatographic purification method was carried out to obtain a concentrated mixture of yuanhuacine and cis 2''/3'' isomer of gnididin on an Xbridge® preparative C18 OBD™ (10 mm $\times$ 250 mm, 5 μ m) column where solvent A consisted of H₂O (0.1% formic acid) and solvent B consisted of MeOH (0.1% formic acid). A fixed flowrate of 5 mL/min was used with the elution method optimised as follows: an initial hold at 15% solvent B (0–1 min) before a linear increase to 100% solvent B (1–13 min) before an isocratic hold at 100% solvent B for 2 min (13–15 min) before reverting to the starting conditions (15–15.5 min) and re-equilibrating the column (15.5–19.5 min). The UV scan range was set at 210–400 nm.

Further purification of the concentrated mixture (35 mg) to obtain yuanhuacine and cis 2''/3'' isomer of gnididin was carried out using the same chromatographic system and column. The gradient elution to obtain the compounds was as follows: solvent A consisted of H₂O (0.1% formic acid) and solvent B consisted of MeOH (0.1% formic acid). A fixed flowrate of 5 mL/min was used with the elution method optimised as follows: an initial hold at 15% solvent B (0–1 min) before a linear increase to 100% solvent B (1–17 min) before an isocratic hold at 100% solvent B for 3 min (17–20 min) before reverting to the starting conditions (20–20.5 min) and re-equilibrating the column (20.5–24.00 min). The UV scan range was set at 210–400 nm.

The data was collected using MassLynx4.3 (Waters, USA) software and the ionization was operated in the positive mode. The probe temperature and source temperature were set at 600 °C and 120 °C, respectively. The capillary and cone voltage were set to 800 and 10 V, respectively. Data was collected between 300 and 1000 m/z. The pure compounds were collected using a fraction collector and subsequently combined and dried using an EZ-2

Genevac. The compounds were later analysed by UPLC-QTOF-MS and their structures confirmed using NMR and MS spectra.

3.6.7. Chemical profiling using UPLC-QTOF-MS of fractions and compounds

The chemical profiling of active fractions and compounds was performed using a Waters Acquity UPLC system (Waters Corp., MA USA) equipped with a binary solvent delivery system. The auto sampler was operated using MassLynx 4.1 software (Waters Inc., Milford, Massachusetts, USA). Instrument calibration was performed using sodium formate solution over a mass range of 50-1200 Da to an absolute mass accuracy of <0.5 mDa. The separation of compounds was achieved using a Waters BEH C18 (1.7 μ m) reverse phase column with particle size 2.1 mm $\times$ 100 mm. The mobile phase consisted of water in 0.1 % formic acid (solvent A) and methanol in 0.1 % formic acid (solvent B). The gradient elution was carried as follows: 3% (0-1 min) and was kept constant at 100% B between (0.3-14 and 14-16 min) and later optimized to 3% B (16.50-20.00 min). The flow rate was maintained at a 0.4 ml/min with an injection volume of 5 μ l with a constant column temperature of 40 $^{\circ}$ C. The following MS source parameters were set for both positive and negative mode: source temperature 100 $^{\circ}$ C, sampling cone 15 V, extraction cone 4.0 V, desolvation temperature 400 $^{\circ}$ C, cone gas flow 10.0 l/h, desolvation gas flow 700 l/h, capillary 2.0 kV. Both positive and negative modes were obtained, however the results were analysed using the positive mode as the higher intensity peaks were obtained from this mode.

3.6.8. Structure elucidation using NMR spectroscopy

The structure elucidation of isolated compounds was carried out using NMR spectroscopy. The ^{1} H, ^{13} C and 2D NMR spectral data were acquired on a Bruker 500 MHz spectrophotometer at 25 $^{\circ}$ C with Prodigy cold probe, (^{1} H MHz and ^{13} C at 500 MHz). The

chemical shifts were reported in ppm, referenced to residual solvent resonances (CDCl_3 δ_{H} 7.26, δ_{C} 77.16 ppm) for deuterated chloroform CDCl_3 .

3.6.9. *In vitro* anti-HIV activity in the DeCiPhR assay

The HIV inhibition activity of the microfractions and compounds against pNL-NF plasmid was tested in the DeCiPhR assay. The microfractions were screened for their anti-HIV activity at a dual point concentration of 2.5 and 0.25 $\mu\text{g/ml}$. The compounds anti-HIV activity was tested at seven different concentrations of 5 $\mu\text{g/ml}$ to 0.008 $\mu\text{g/ml}$ following a 2-fold serial dilution. The known inhibitor efavirenz was used as a positive control. A detailed procedure of the assay explained in section 2.8, Chapter 2.

3.6.10. Latency reversal activity of isolated daphnane diterpenoids.

The Jurkat-derived, J-Lat 9.2 cells containing an *env* proviral genome were used to evaluate the latency reversal, viability and cellular activation of the isolated compounds in a dose response manner. Latency reversal activity was evaluated in parallel with a PKC activator prostratin and an HDAC inhibitor and was measure by GFP expression. A detailed latency reversal activity procedure is shown in Supplementary data 1.

3.6.11. *Ex vivo* anti-HIV activity of yuanhuacine A in human isolated peripheral blood mononuclear cell

The *ex vivo* anti-HIV activity of yuanhuacine A in peripheral mononuclear cells (PBMCs) was investigated using a subtype C viral strain from Ethiopia was carried out by the Southern Research Organisation, project number 16174.01 (See supplementary data 1). The compound was tested at four different concentrations (0.001 to 0.03 $\mu\text{g/ml}$) following a 3-fold serial dilution. The nucleoside analogue reverse transcriptase inhibitor azidothymidine (AZT) was used as a positive control. Furthermore, the cytotoxicity of the compound was determined at nine different concentrations (0.001 to 10.0 $\mu\text{g/ml}$) following a 3-fold serial dilution.

3.6.12. The human ether-a-go-go related gene (hERG) activity of yuanhuacine A

The human ether-a-go-go gene (hERG, Kv11.1) K⁺ channel plays an important role in cardiac electrophysiology and in drug safety.^[3] The inhibition of hERG is reported to be the most common mechanism for numerous non-anti-arrhythmic compounds that produce the Torsade's de Pointes (TdP) associated with QT prolongation.^[3,21] This necessitates the investigation of *in vitro* drug-hERG interactions as it has become an important part of modern safety pharmacology.^[3,21]

The hERG inhibition activity of yuanhuacine A was investigated with the assistance of Metrion Biosciences. Yuanhuacine A was screened in an automated QPatch clamp assay using a four-point concentration of 0.02, 0.2, 2 and 20 µg/ml. A calcium channel blocker verapamil was used as a reference compound and was tested at 0.3, 1.0, 3.0 and 10 µM. (See Supplementary data 3).

3.6.13. *Ex vivo* latency reversal activity of isolated daphnane diterpenoids

The compounds yuanhuacine A, yuanhuacine, gnididin and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin were tested for their ability to reverse HIV latency using CD4⁺ T cells (primary isolates) obtained from three HIV-1 infected donors. The donors were on stably suppressive cART (< 50 copies/mL of plasma viral load) for at least 3 years following informed written consent. These participants were recruited in accordance with human subject research guidelines of the United States Department of Health and Human Services under the supervision of the Wistar and Philadelphia FIGHT institutional review boards. The primary CD4⁺ T cells were isolated from PBMC using the EasySep Human CD4⁺ T cell Enrichment Kit (STEM-CELL, Vancouver, Canada) and cultured in R20+ media, which was identical to R10+ media described above except for addition of 20% FBS.

The primary cell viability and latency reversal of the compounds yuanhuacine A, yuanhuacine, gnididin and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin was performed.

Briefly, 2×10^6 cells were treated with 0.1% DMSO, test agent, or anti-CD3/CD28 dynabeads (Invitrogen, Carlsbad, CA, USA) in a 1:1 ratio for 24 hours. Following incubation, live cells were counted visually by trypan blue staining. Viral RNA was extracted from culture supernatants using a QIAmp Viral RNA Mini kit (Qiagen, Germantown, MD, USA) and subjected to quantitative RT-PCR as described previously using a C1000 Thermal Cycler and CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA).^[18] The limit of detection of supernatant viral RNA was 10 copies/mL as determined through repeating end point detection from serial dilution of the AcroMetrix HIV-1 Panel (ThermoFisher). (See Supplementary data 1 as provided for by Prof Ian Tietjen of the Wistar Institute, USA)

3.7. Results and discussion

3.7.1. Localised microfractionation using HPLC

The localization of anti-HIV active compounds from the DCM extract (300 µg) by HPLC microfractionation was repeated three times and resulted in the collection of 30 microfractions in each 96 deep-well plate. The deep-well plates were dried using a speedvac, an estimation of the quantity in each well was made to be approximately 10 µg for each microfraction. The microfractions were screened *in vitro* for anti-HIV activity and the bioactive microfractions were further chemically analysed by UPLC-MS.

3.7.2. *In vitro* anti-HIV activity of HPLC microfractions

The *in vitro* anti-HIV activity and cytotoxicity of the 30 microfractions at 2.5 µg/ml is shown in Figure 3.4. The microfractions showed inhibition activities of $52.0 \pm 0.9\%$, $71.0 \pm 5.9\%$, $83.9 \pm 9.6\%$, $103 \pm 2.1\%$, 101 ± 2.0 and $91.6 \pm 2.8\%$ corresponding to deep-well plate, numbered 25, 26, 27, 28, and 29, respectively. The fractions exhibited some cytotoxicity (<50%) at this test concentration which could be due to the presence of active compounds in high concentrations.

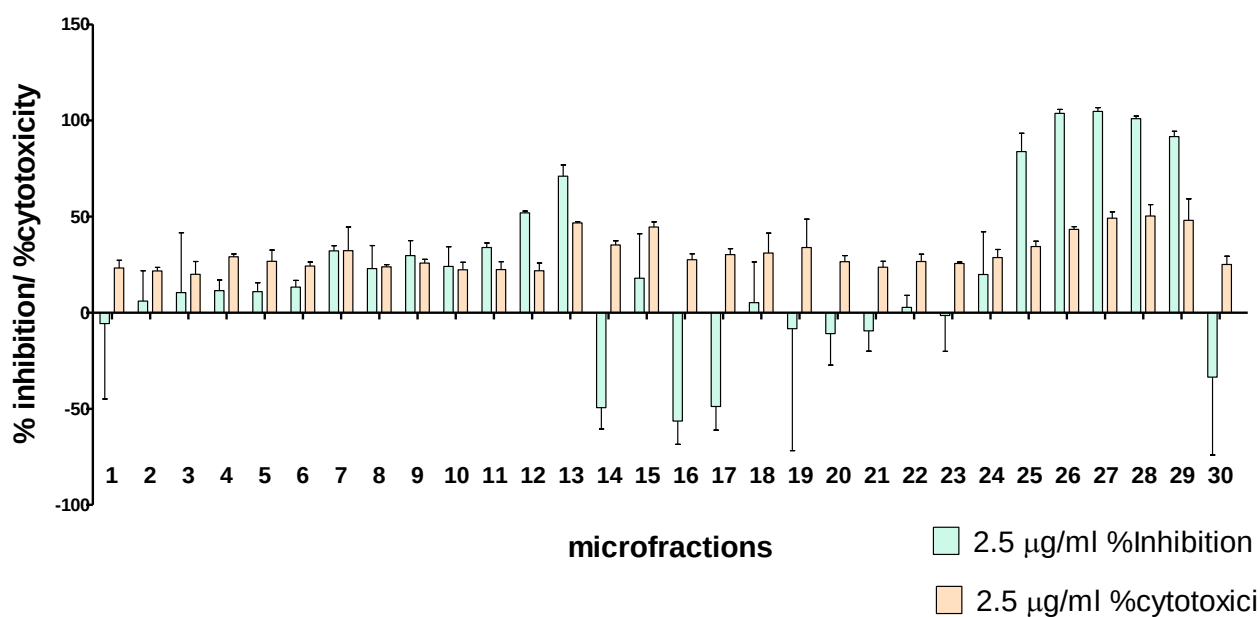


Figure 3.4: Anti-HIV activity of microfractions collected in 30 minutes in deep-well (1-30) tested at 2.5 µg/ml.

The microfractions were further evaluated for their anti-HIV activity at 0.25 µg/ml however none showed any anti-viral replication properties as shown in Figure 3.5. The inactivity of the fractions at low test concentration could have been due to the dilution of active compounds in the microfractions due the estimation of the quantities per well at 10 µg as these may have been much lower leading to a sub-therapeutic concentration.

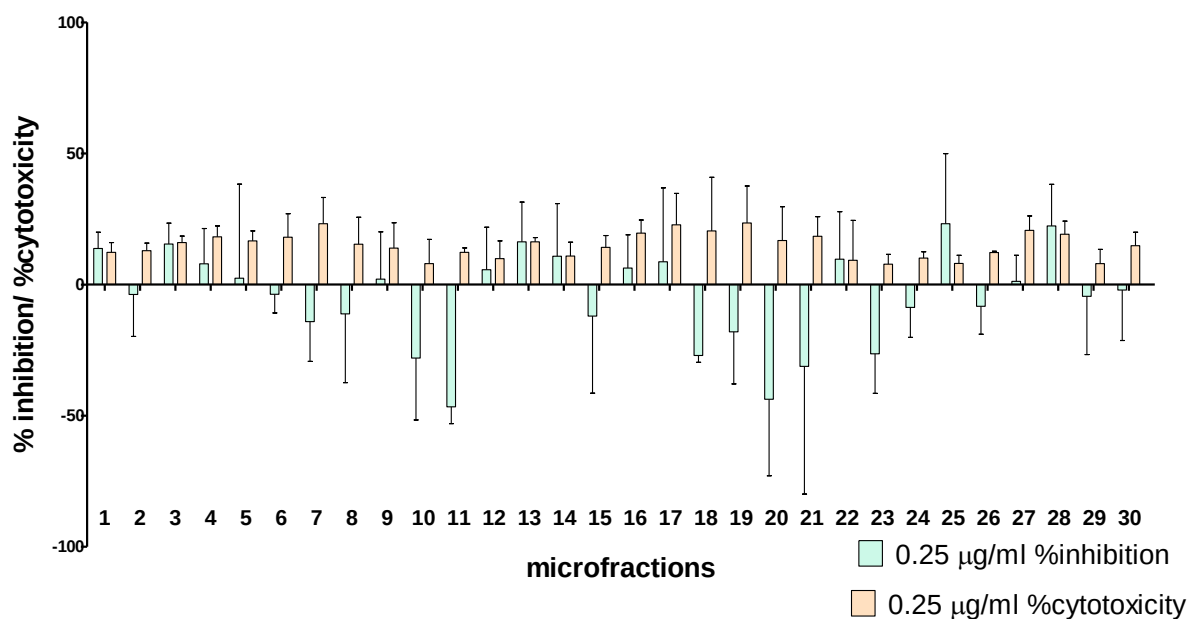


Figure 3.5: Anti-HIV activity of microfractions collected in 30 min in deep-well from minute (1-30) and screened at 0.25 µg/ml.

The anti-HIV activity of the efavirenz is shown in Figure 3.6. The results show that efavirenz inhibited the virus 76.84% when tested at 0.029 µg/ml. This activity is comparable to the anti-HIV activity shown by the microfraction when screened at 2.5 µg/ml

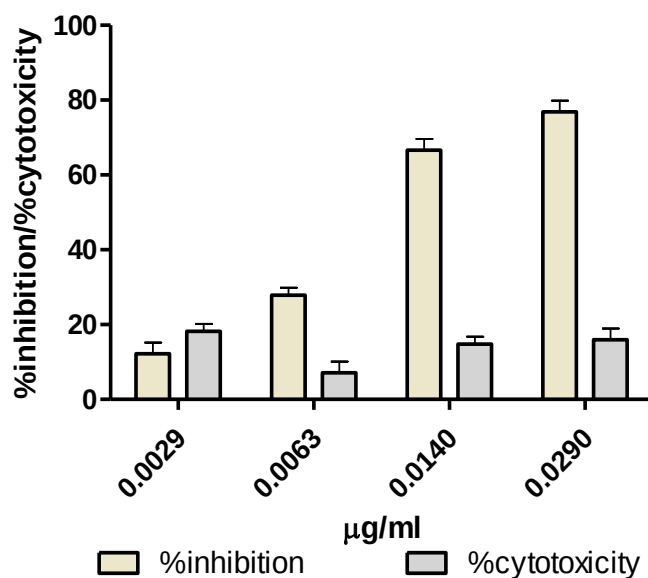


Figure 3.6: Anti-HIV activity of efavirenz at different concentrations.

All active fractions were further chemically profiled to identify the possible active compounds in the microfractions. The microfractionation of the DCM extract using HPLC was a success as the activity of the extract was localised. The HPLC based activity profiling as adapted from Hamburger et al., (2009) has provided to be a very useful tool in the fast tracking of compound with anti-HIV activity in the microfraction. Furthermore, the technique has proven to be a cost effective as only micro scale quantities of plant extracts were used in a short period of time.

3.7.3. UPLC-MS-QTOF analysis of the active microfractions

The chemical profile of the active microfraction in the positive ionization mode is shown in Figure 3.7. The chromatograms showed two common peaks in all bioactive microfraction sample that were not present in the solvent blank. These peaks were detected at m/z 637.3046 at retention time 12.52 minutes and at m/z 425.2883 at retention time 12.76 minutes which were tentatively identified as acetylsucrose and 1-butyl-3-ethyl-8-methoxy-4-methyl-2-phenyl-5-propan-2-ylanthracene, respectively, and were not detected in the DCM extract. Moreover, the daphnane diterpenoids which were identified in the DCM extract were not detected in these microfractions. It was then concluded that the compounds were very dilute in each microfraction, as a result of which, the microfractions were combined and further analysed by UPLC-QTOF-MS.

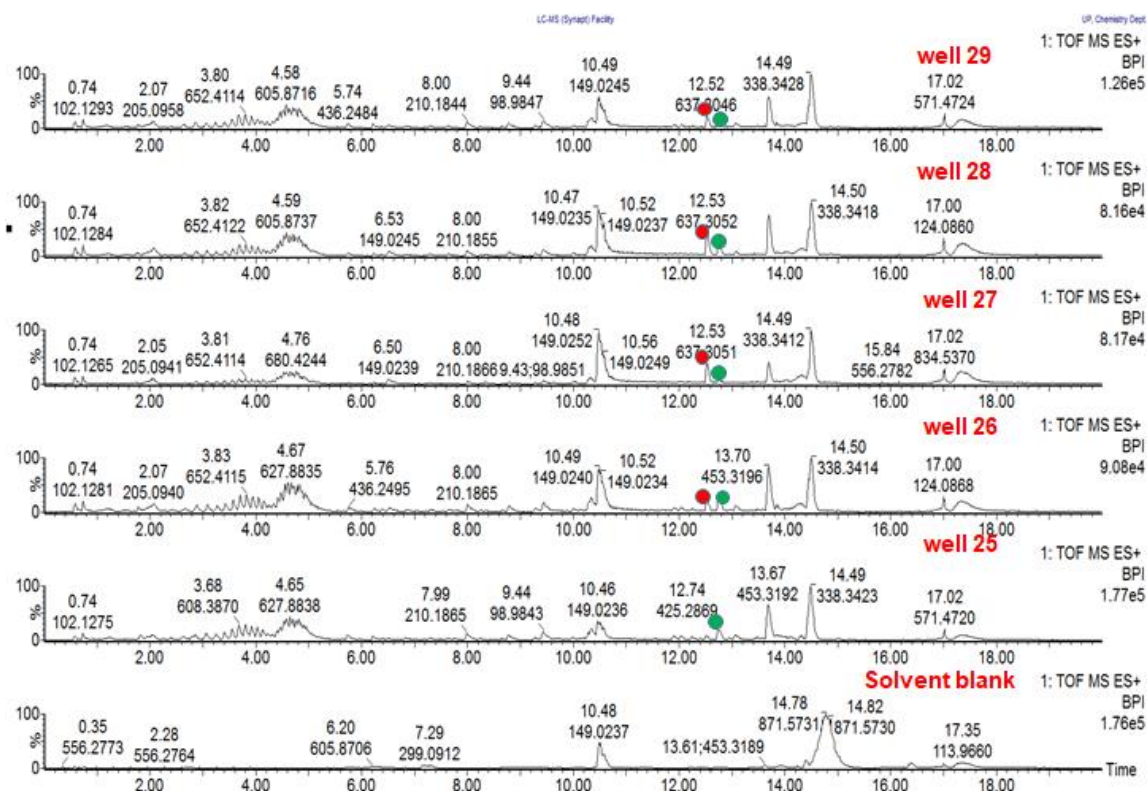


Figure 3.7: UPLC-QTOF-MS analysis of the active microfractions in well (25, 26, 27, 28 and 29) and the blank in ESI positive mode.

The chemical analysis of the combined microfractions (well 25, 26, 27, 28 and 29) using UPLC-QTOF-MS is shown in Figure 3.8. The two compounds previously detected in the individual microfractions were suppressed in the combined fraction. This meant that the compounds were not responsible for the anti-HIV activity in the micro-fractions. The presence of daphnane diterpenoids based on literature eluting between 11-13 minutes in the chromatogram, was observed.^[6] For peak 1 the $[M+H]^+$ ion observed at m/z 621.2689 at retention time 11.31 minutes was previously identified as yuanhuacine A. For peak 2 the $[M+H]^+$ ion observed at m/z 647. 2863 at retention time 11.58 minutes was previously identified as yuanhuajine. For peak 3, the observed $[M+H]^+$ at m/z 647.2863 at retention time 12.03 minutes, was previously identified as gniditrin. The $[M+H]^+$ ion observed for peak 4 eluting in 12.42 minutes at m/z 649.3020 was detected as the most intense daphnane diterpenoids in the fraction and was previously reported as yuanhuacine in the DCM extract.^[8] The combination of the microfractionation enhanced the detection of the anti-HIV active daphnane diterpenoids.

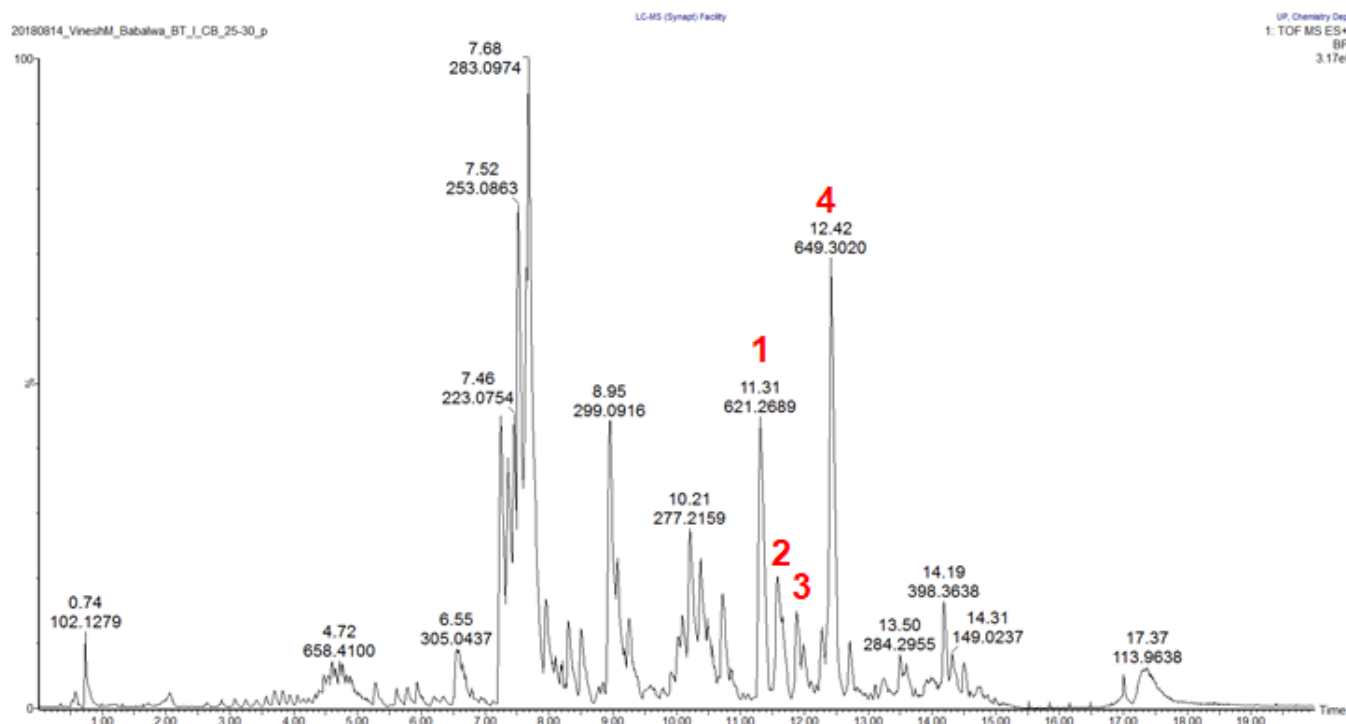


Figure 3.8: UPLC-MS profile of combined active microfractions eluting between 11.31 to 13.0 minutes in ESI positive mode.

Yuanhuacine and yuanhuacine A were previously reported in *Gnidia polycephala* as one of the minor daphnane diterpenoids.^[3] The compounds, yuanhuacine A and yuanhuacine in *G. sericocephala* fractions were detected as the most intense peaks compared to other daphnane diterpenoids. The presence of these compounds in these fractions supported the anti-HIV activity seen in the microfractions. The HPLC based activity localization has provided a very effective tool in early compound dereplication.

3.7.4. HPLC time based purification of the major peaks in the DCM extract

The targeted purification of the major peak in the DCM extract (shown in Chapter 2, Figure 2.29) was carried out to obtain the chemical markers of the extract. This was performed using a flash chromatography on a BUCHI Pure C-815-Flash system (Buchi, Flawil, Switzerland) fitted with a UV detector and an ELSD. The extract (20 g) was injected onto a Buchi EcoFlex silica cartridge (220 g, 50 μ m). An isocratic MeOH : H₂O (50:50) solvent system with the UV

wavelengths set at 249 and 270 nm for detection was used. A very intense smooth peak was detected between 20-22 minutes of the run, repeated injections of the crude extract into the semipreparative RP column resulted in the collection of the peak. The fraction (3 mg) was obtained after drying using a high speedvac. The chemical analysis of the fraction using UPLC-QTOF-MS and NMR showed a presence of two compounds in a 1:2 ratio. Further purification of the compounds was done using an HPLC-MS-SPE system

3.7.5. UPLC-MS-QTOF analysis of the major peaks obtained by HPLC time based fractionation

The chemical profile of the mixture of the major compounds obtained by HPLC time based fractionation is shown in Figure 3.9 and their MS/MS fragments shown in Table 3.1. The $[M+H]^+$ observed for peak 1 with m/z 305.0665 corresponded to molecular formula $C_{18}H_9O_5$ eluting at 6.54 minutes and was previously identified in the DCM extract (Chapter 2, Figure 2.29) as lasioerin a biscoumarin. The $[M+H]^+$ observed for peak 2 with m/z 337.0933 corresponded to molecular formula $C_{19}H_{13}O_6$ eluting at 6.68 minutes was previously identified in the DCM extract (Chapter 2, Figure 2.29) as lasiocephalin.^[3,22,23]

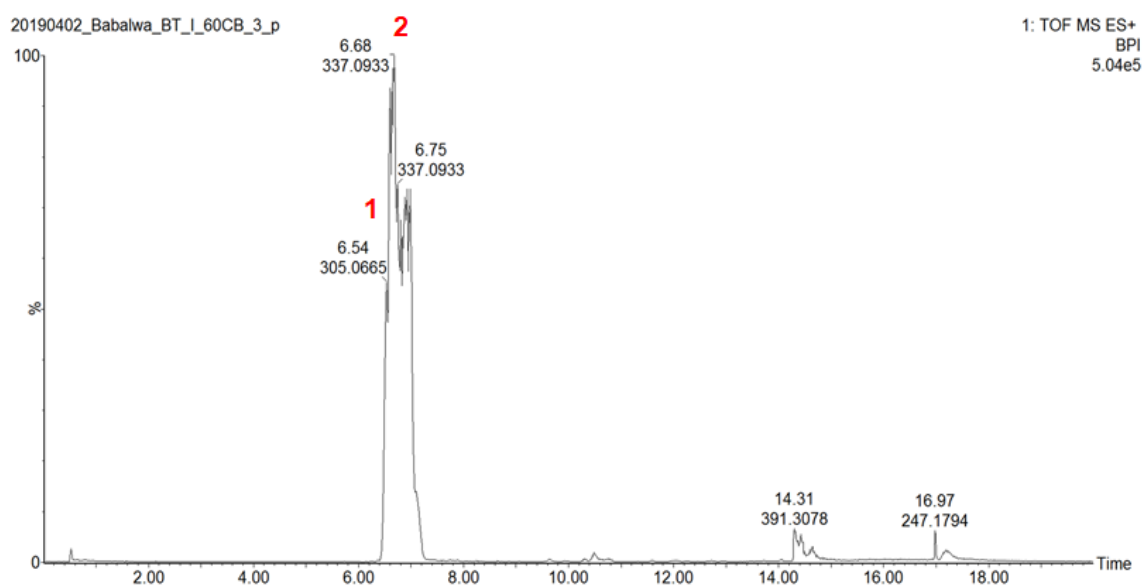


Figure 3. 9: UPLC-MS-QTOF profile of the major peak of the DCM extract of *G. sericocephala* roots. A mixture of two compounds eluting from 6.54 and 6.68 minutes in ESI positive mode.

Table 3.1: Chemical profile of the major peaks of *G. sericocephala* root extract.

Peak No.	Observed [M+H] ⁺	Theoretical [M+H] ⁺	Mass error ppm	Molecular formula	MS/MS fragmentation	Compound name
1.	305.0665	305.0450	-3.6	C ₁₈ H ₈ O ₅	249.0715	Lasioerin
					221.0756	
					205.0740	
					165.0796	
2.	337.0933	337.0699	-3.9	C ₁₉ H ₁₂ O ₆	321.0547	Lasiocephalin
					265.0632	
					146.0423	

The MS/MS spectra of the peak 1 (Supplementary data 5) showed a fragmentation ion at m/z 249.0715 due to the loss of C₃H₂O followed by a loss of two water molecules to give fragment ion at m/z 221.0756. The subsequent loss of two water molecules led to fragment ion at m/z 205.0740 and the fragment ion at m/z 165.0796 was due to a loss of C₅H₅ as shown in Figure 3.10.^[3,6,22,23]

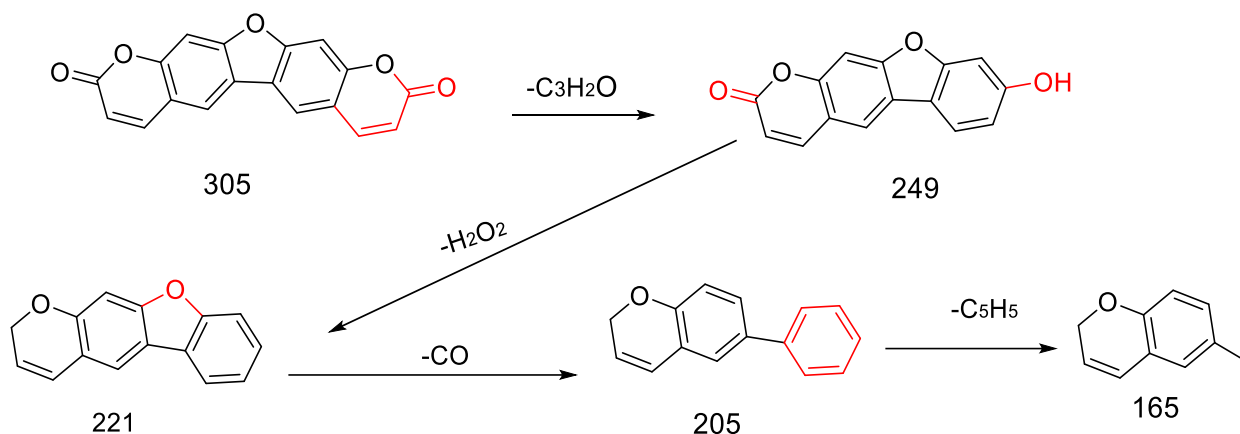


Figure 3. 10: The MS/MS fragmentation pattern for lasioerin ion ESI positive mode.

The MS/MS spectra (Supplementary data 6) fragmentation pattern of lasiocephalin is described in detail in Figure 3.11. The loss of a methyl group in the compound led to fragment ion at m/z 321.0547. The subsequent loss of two water molecules followed by loss of a carbon monoxide led to fragment ion at m/z 265, which was further fragmented as shown by fragment ion at m/z 146 due to the loss of $C_7H_{10}O$. Due to this the compound was tentatively identified as lasiocephalin.^[24]

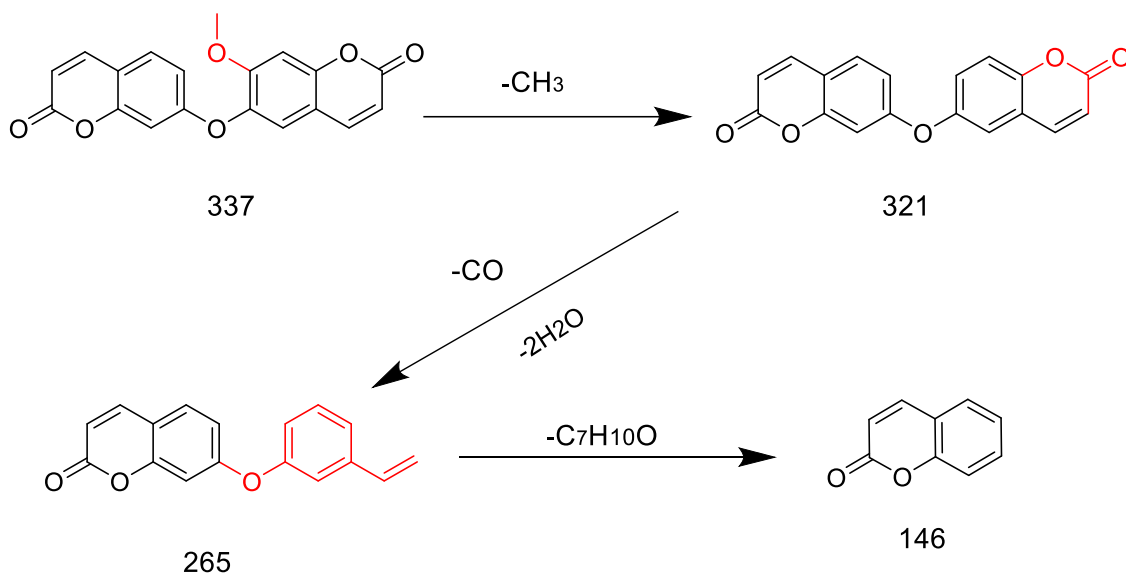


Figure 3.11: MS/MS fragmentation pattern of lasiocephalin in ESI positive mode.

3.7.6. HPLC-MS-SPE purification of the major peaks

The two peaks were further purified using an HPLC-MS-SPE system for future use as chemical markers of *G. sericocephala* roots. The HPLC showed two separate peaks with UV absorptions of 270 and 315 nm, for peak 1 and peak 2, respectively. Each of the two peaks was trapped on an SPE cartridge using a multitrapping function. The adsorbed compounds were later eluted from the cartridges with acetonitrile into vials and dried on an EZ-2 Genevac. The compounds 1 (1.5 mg) and 2 (0.5 mg) were further characterised by UPLC-QTOF-MS to determine their purity. The purity of the compounds was confirmed based on the profile of the major peaks at m/z 337.0922 and m/z 305.0589 in ESI positive mode as shown in Figure 3.12 and 3.13, respectively.

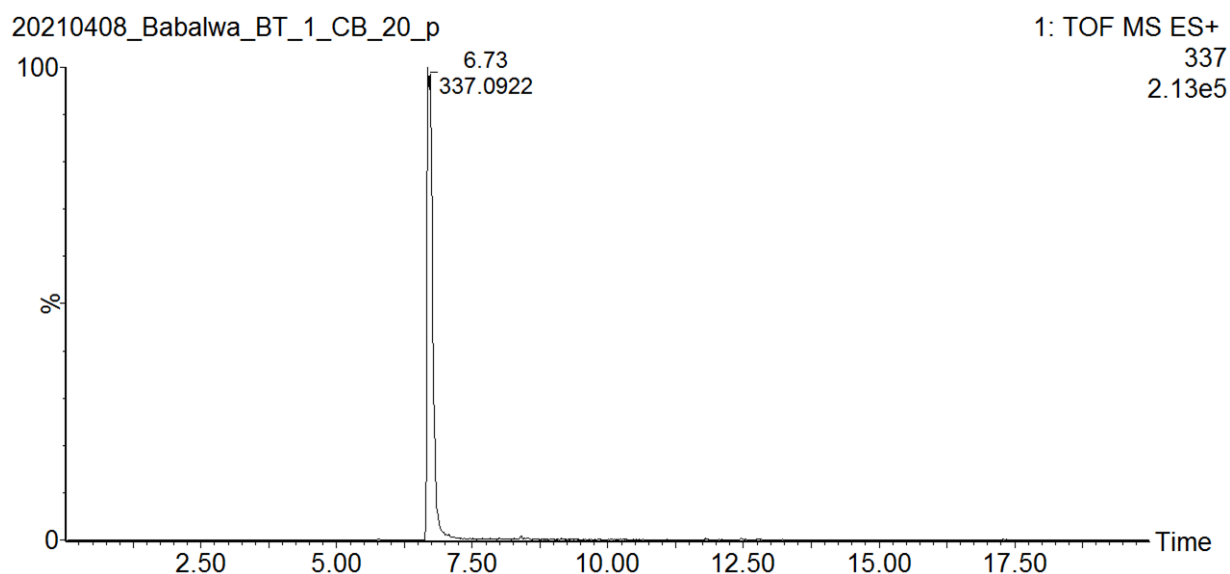


Figure 3.12: UPLC-MS-QTOF profile of lasiocephalin one of the chemical markers of *G. sericocephala* DCM extract with retention time of 6.73 minutes in ESI positive mode.

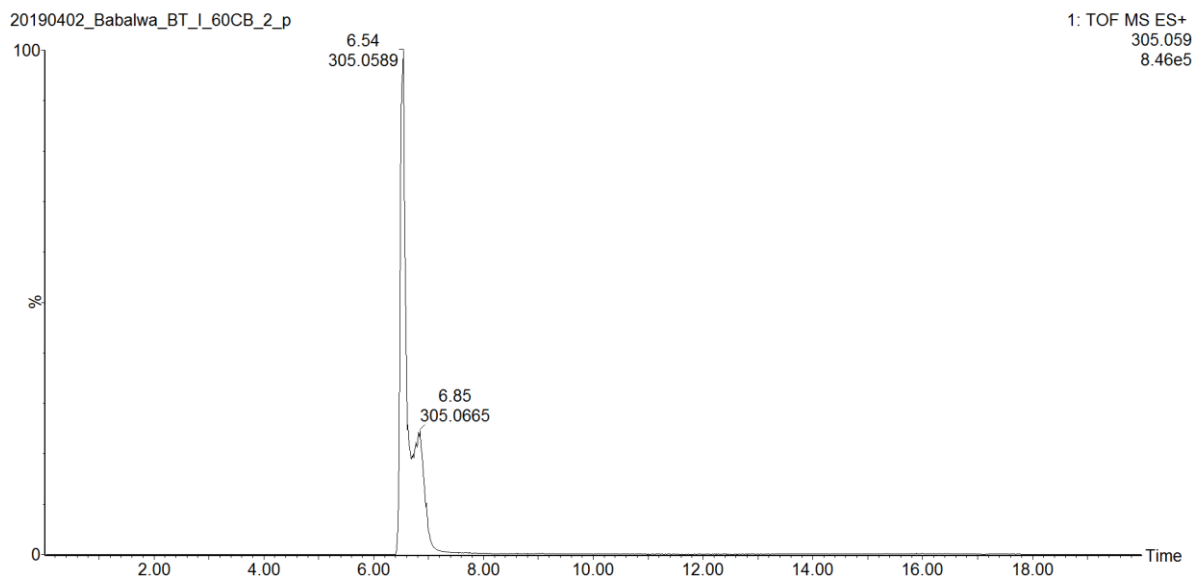


Figure 3.13: UPLC-MSQTOF profile of lasioerin the second chemical marker of *G. sericocephala* DCM extract with a retention time of 6.54 minutes in ESI positive mode.

3.7.7. *In vitro* anti-HIV activity of the two isolated major compounds

The compounds were simultaneously tested *in vitro* for their anti-HIV activity with the microfractions using the DeCiPhR assay. Both compounds did not exhibit any anti-HIV activity at all test concentrations as shown in Figure 3.14. The inactivity of the two biscoumarins also confirmed the anti-HIV activity in the extract was due to the presence of daphnane diterpenoids resulting in the targeted isolation of the diterpenoids.

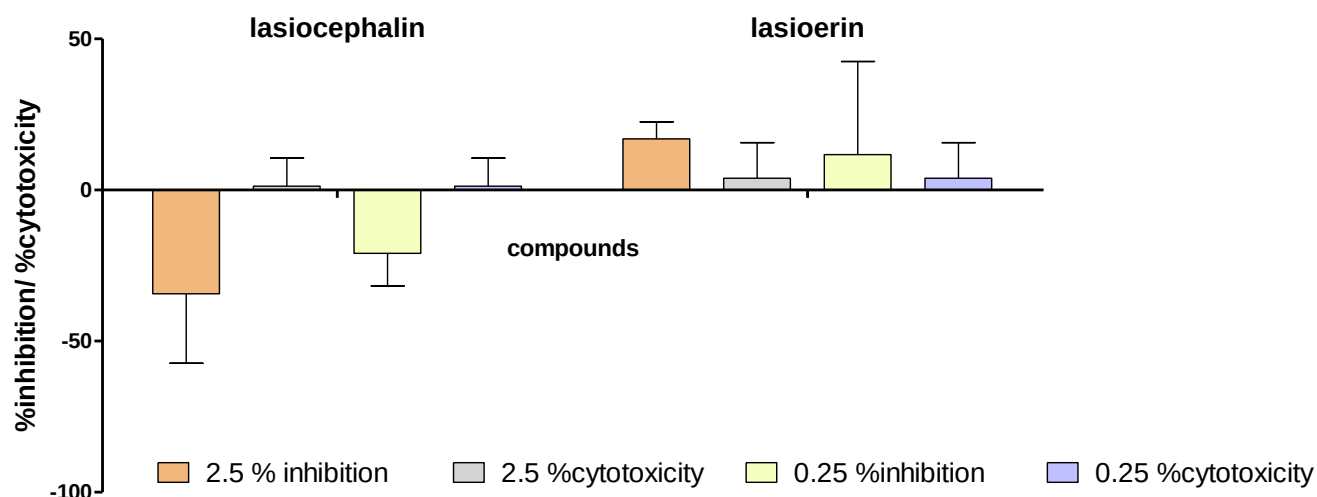


Figure 3.14: *In vitro* anti-HIV activity of lasioerin and lasiocephalin at 2.5 and 0.25 µg/ml.

3.7.8. Fractionation using silica gel column chromatography

The preparative purification of the active daphnane diterpenoids was carried out using silica gel column chromatography of the DCM leaf extract (16 g). A gradient solvent system resulted in the collection of 22 fractions which were analysed by TLC. Fractions with similar R_f values were combined to give 13 fractions as shown in Figure 3.15 and chemically analysed using UPLC-TOF-MS. The two fractions, BT-CB22-6 (250 mg) and BT-CB22-11 (28 mg) were the only fractions that showed the presence of daphnane diterpenoids after chemical analysis using UPLC-QTOF-MS.

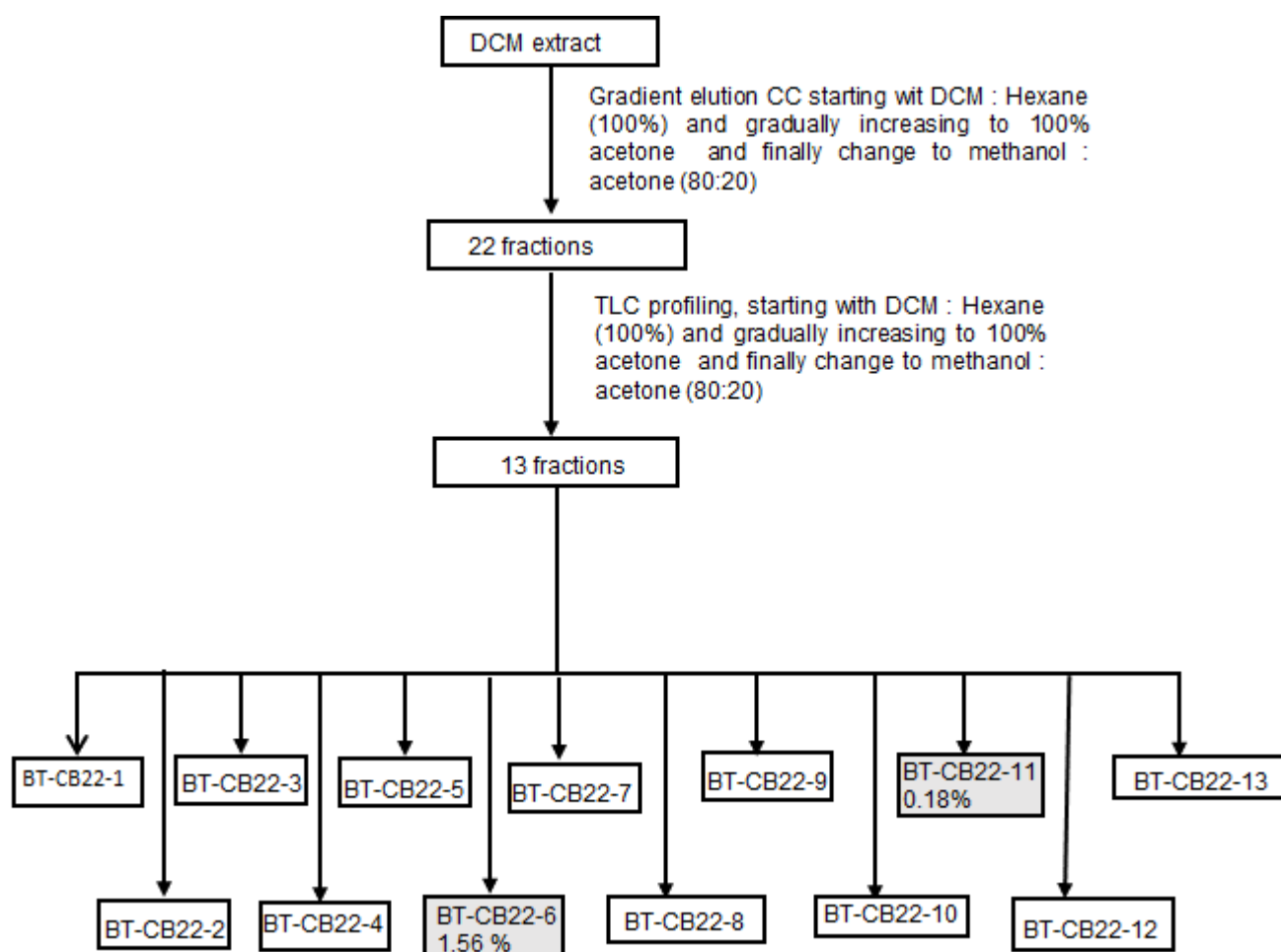


Figure 3.15: Flow diagram for the fractionation of the root extract of *G. sericocephala* using silica gel column chromatography. The highlighted fractions contained daphnane diterpenoids with their obtained %yields shown.

3.7.9. UPLC-QTOF-MS analysis of fractions obtained using silica column chromatography

The chemical profile of the fractions BT22-6 (chromatogram A) and BT22-11 (chromatogram B) obtained using column chromatography is shown in Figure 3.16. Chromatogram A revealed the presence of molecular ion $[M+H]^+$ at m/z 647.31.56 eluting at 14.41 minutes which corresponded to peak 3 of the combined microfractions and a molecular ion $[M+H]^+$ at m/z 649.3286 eluting at 14.56 minutes which corresponded to peak 4 of the combined microfractions (Figure 3.8). Chromatogram B also revealed the presence of daphnane

diterpenoids with molecular ion $[M+H]^+$ with m/z 621.2996 eluting at 14.37 minutes which corresponded to peak 1 of the microfractions (Figure 3.8) and $[M+H]^+$ with m/z 649.3266 eluting at 14.70 minutes corresponding to peak 4 of the microfractions (Figure 3.8). However, only two peaks with m/z 621.2996 and m/z 649.3286 were successfully purified in the fractions. In order to obtain all the compounds flash chromatography was further used.

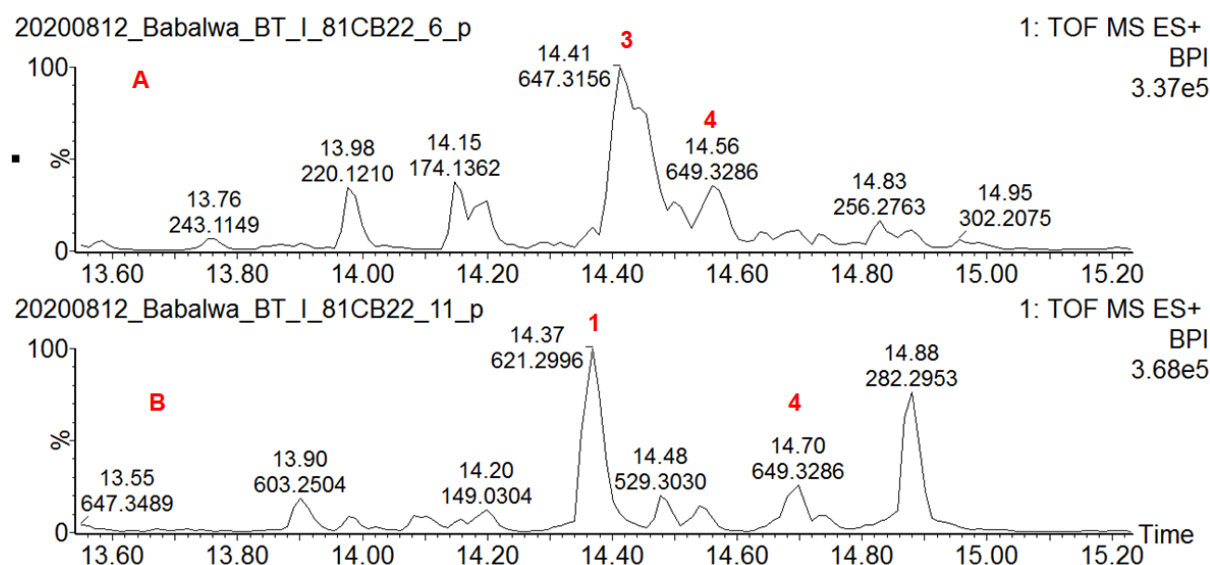


Figure 3.16: UPLC-MS profile of active fractions obtained by silica gel column chromatography.

3.7.10. Fractionation using flash chromatography

The fractionation of the DCM extract using flash chromatography was done to obtain the daphnane diterpenoids which were not able to be purified by silica gel column chromatography using a flash chromatography on a BUCHI Pure C-815-Flash system (Buchi, Flawil, Switzerland) fitted with a UV detector and an ELSD. The extract (20 g) was injected onto a Buchi EcoFlex silica cartridge (220 g, 50 μ m). The detection and collection profile of the DCM extract is shown in Figure 3.17. The compounds were detected by UV at 215 and 230 nm and the ELSD threshold set at 20 mV. The fractions were combined, dried and weighed based on the colours shown by each test tube and chemically analysed using UPLC-QTOF-MS. Only fraction BT-8 (150 mg) revealed the presence of the targeted daphnane diterpenoids.

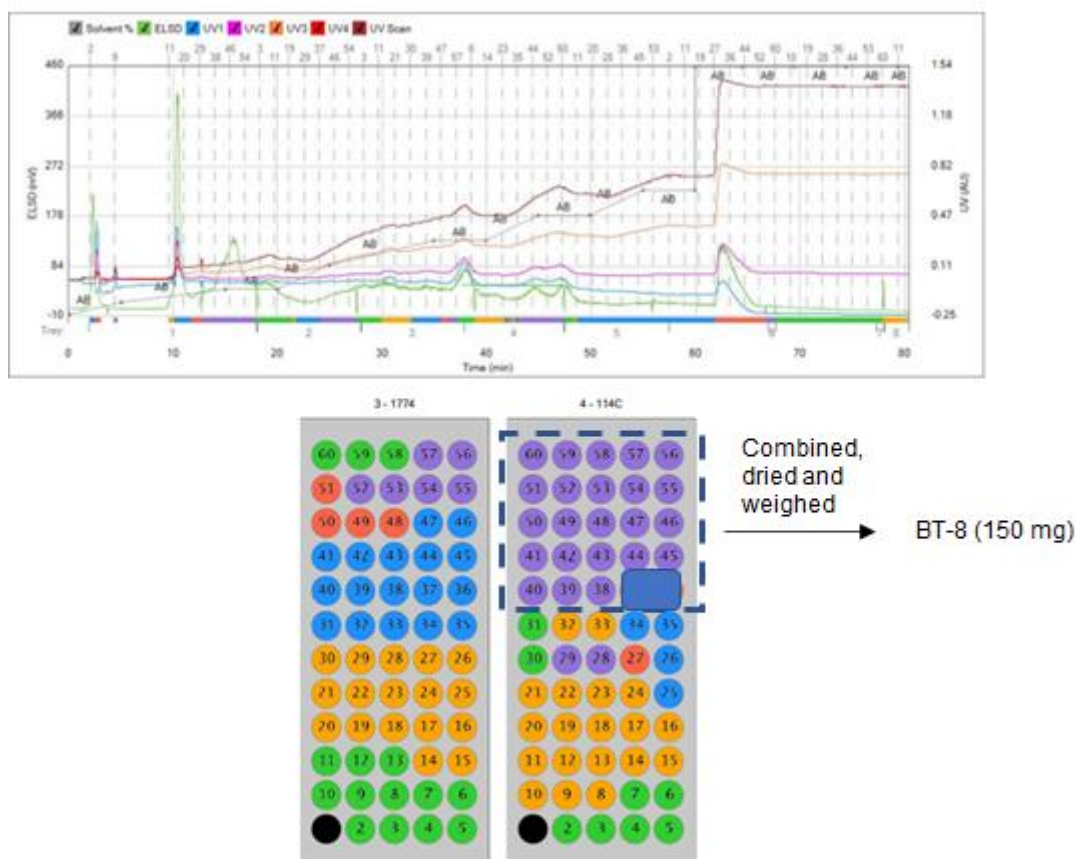


Figure 3.17: Process for the detection and collection of fractions from the DCM extract using flash chromatography

The fractionation of the ethyl acetate fraction using flash chromatography was done to obtain the daphnane diterpenoids using a flash chromatography on a BUCHI Pure C-815-Flash system (Buchi, Flawil, Switzerland) fitted with a UV detector and an ELSD. The fraction (30 g) was injected onto a Buchi EcoFlex silica cartridge (220 g, 50 μ m). The detection and collection profile of the DCM extract is shown in Figure 3.18. The compounds were detected by UV at 215 and 230 nm and the ELSD threshold set at 20 mV. The fractions were combined, dried and weighed based on the colours shown by each test tube and chemically analysed using UPLC-QTOF-MS. Fractions BT-G (200 mg) and BT-H (150 mg) revealed the presence of the targeted daphnane diterpenoids, and the two fractions were combined labelled as BT-GH (350 mg).

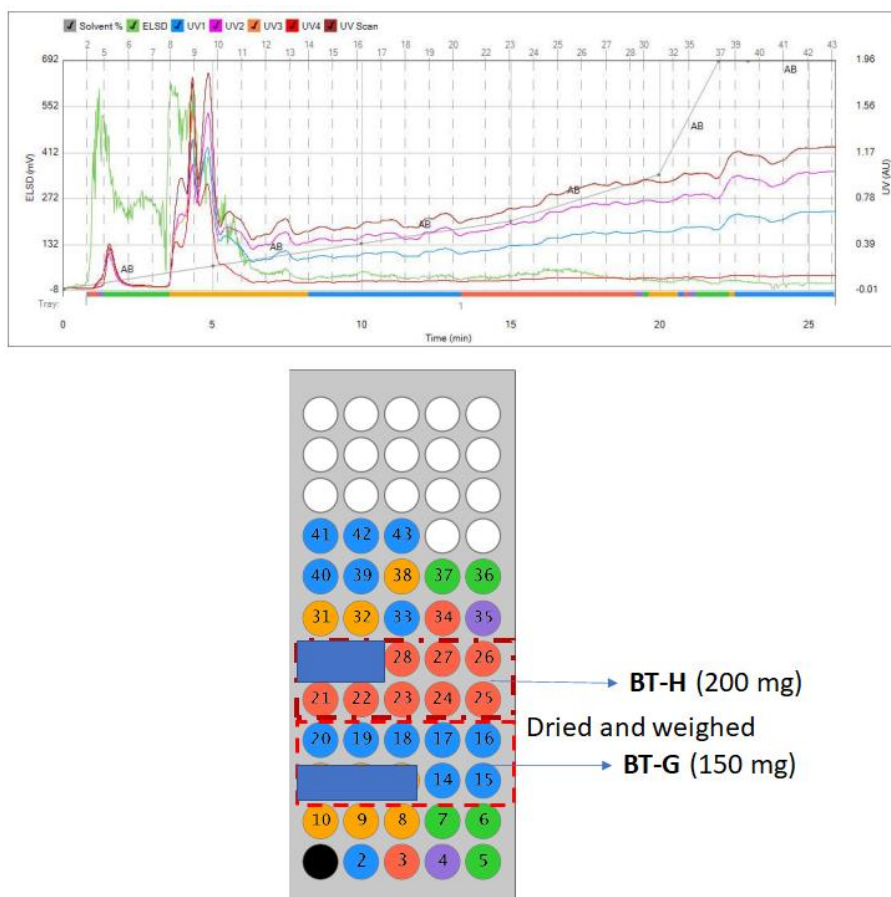


Figure 3.18: Process for the detection and collection of fractions from the ethyl acetate fraction using flash chromatography

3.7.11. UPLC-QTOF-MS analysis of fractions obtained by flash chromatography and HPLC-MS chromatography

The UPLC-MS profile in the ESI positive mode of fraction BT-8 obtained using flash chromatography as shown in Figure 3.19 revealed the presence of daphnane diterpenoids eluting between 11-13 minutes. A molecular ion $[M+H]^+$ at m/z 621. 2958 eluting in 11.25 minutes which corresponded to peak 1 of the combined active microfractions (Figure 3.8) was detected. The molecular ion $[M+H]^+$ at m/z 647.3134 eluting in 11.54 minutes which corresponded to peak 2 and $[M+H]^+$ ion at m/z 647.3134 eluting in 11.99 minutes which corresponded to peak 3 as well as the $[M+H]^+$ at m/z 649.3267 eluting at 12.44 minutes which

corresponded to peak 4 of the microfractions (Figure 3.8). The fraction was further purified using a mass directed approach on a preparative reverse phase HPLC.

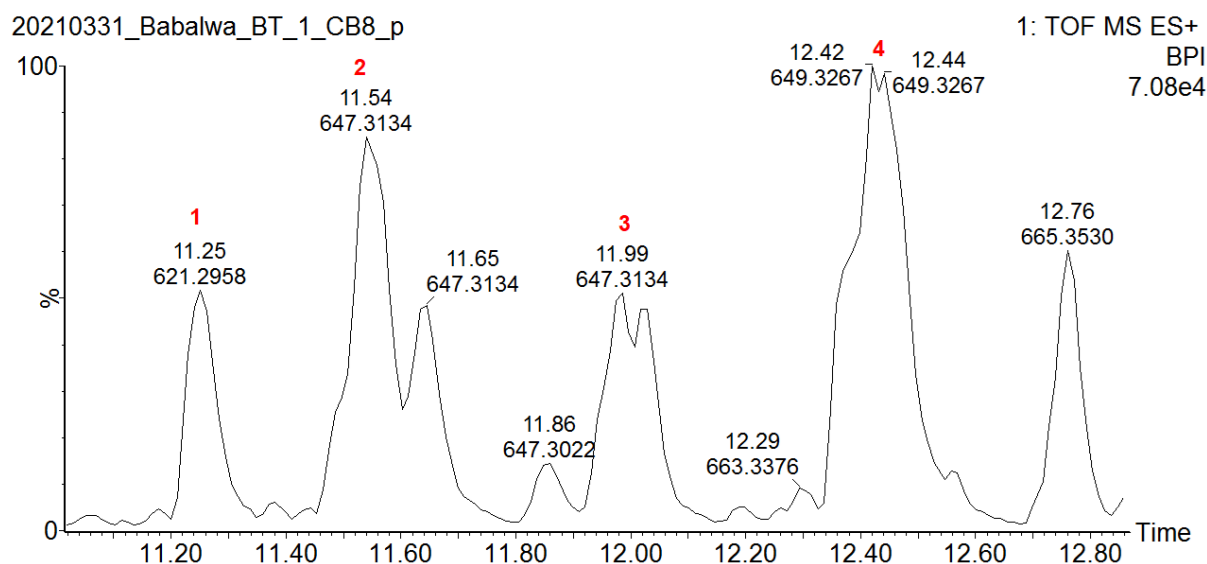


Figure 3. 19: UPLC-MS profile of active fractions obtained by flash chromatography eluting between 11.20- 12.45 minutes

The UPLC-MS profile of fraction BT-GH-1 obtained using a preparative HPLC-MS in the ESI positive mode as shown in Figure 3.20 revealed the presence of two daphnane diterpenoids (peak 1) eluting at 11.29 min and yuanhuacine (2) eluting at 11.49 min with a molecular mass of 649.2893. A molecular ion $[M+H]^+$ at m/z 649. 2893 which corresponded to peak 1 was tentatively identified as gnididin of the preparative HPLC-MS fraction (Figure 3.8.1) was detected. The molecular ion $[M+H]^+$ at m/z 649.2893 which corresponded to peak 2 was tentatively identified as yuanhuacine (Figure 3.20). The fraction was further purified using a mass directed approach on a preparative reverse phase HPLC to obtain yuanhuacine and gnididin in their pure form.

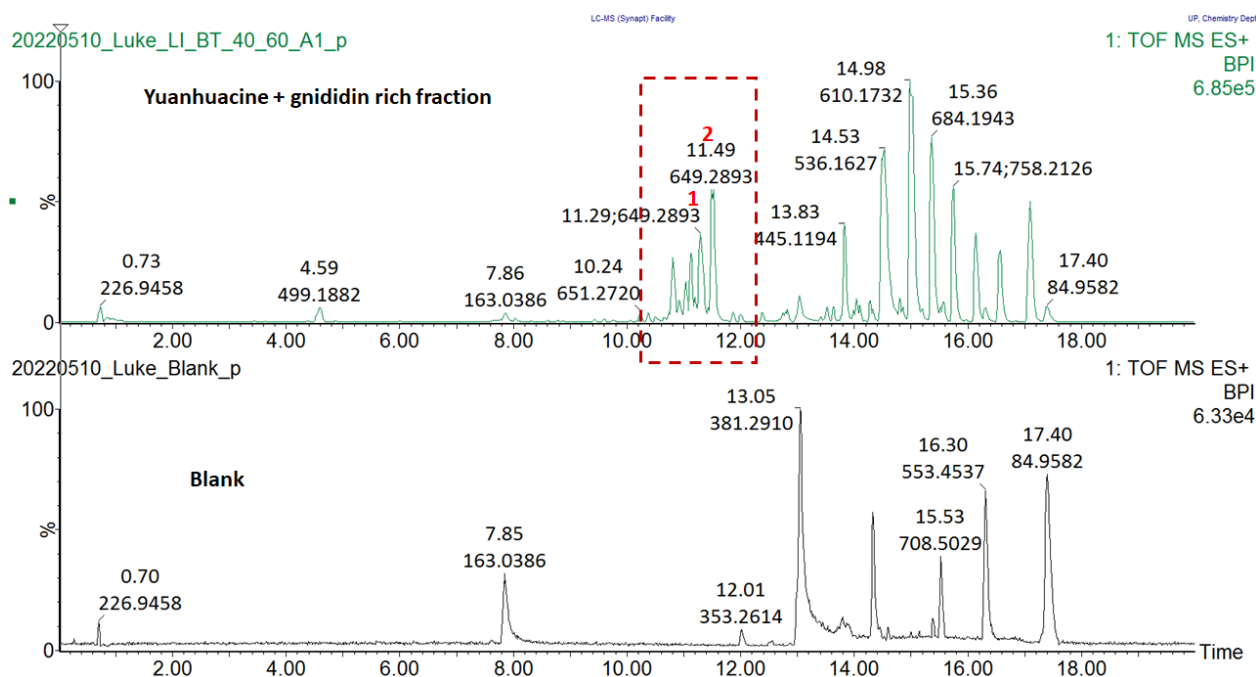


Figure 3. 20: UPLC-MS profile of active fractions obtained by flash chromatography eluting between 11.20- 12.45 minutes

3.7.12. Preparative purification and isolation of compounds from active fractions

The compounds, yuanhuacine A, gniditrin, yuanhuajine as well as the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin were purified by injecting 150 μ l which corresponded to 50 mg of the sample on an Xbridge® preparative C18 OBD™ (19 mm $\times$ 250 mm, 5 μ m) column. The compounds were collected in test tubes using a mass triggered fractionation. The fractions BT22-11 and BT22-6 obtained using column chromatography were combined and were further purified by repeated injection following a mass directed purification on a preparative HPLC. All peaks in the ESI positive mode that corresponded to daphnane diterpenoids with m/z values of 621.2996 (BT8-1), 647.3044 (BT8-2A), 647.3044 (BT8-2B) and 649.3176 (BT8-3) were targeted and collected separately (Figure 3.21). Peaks with the same m/z value were combined and dried using a Genevac- EZ2. The compounds with m/z values of 621.2872 (2.96 mg) and 649.3176 (8.25 mg) as shown in (Figure 3.19) were obtained in pure form and sufficient quantities for further chemical and biological analysis. The purity of compounds was confirmed by UPLC-MS and NMR analysis.

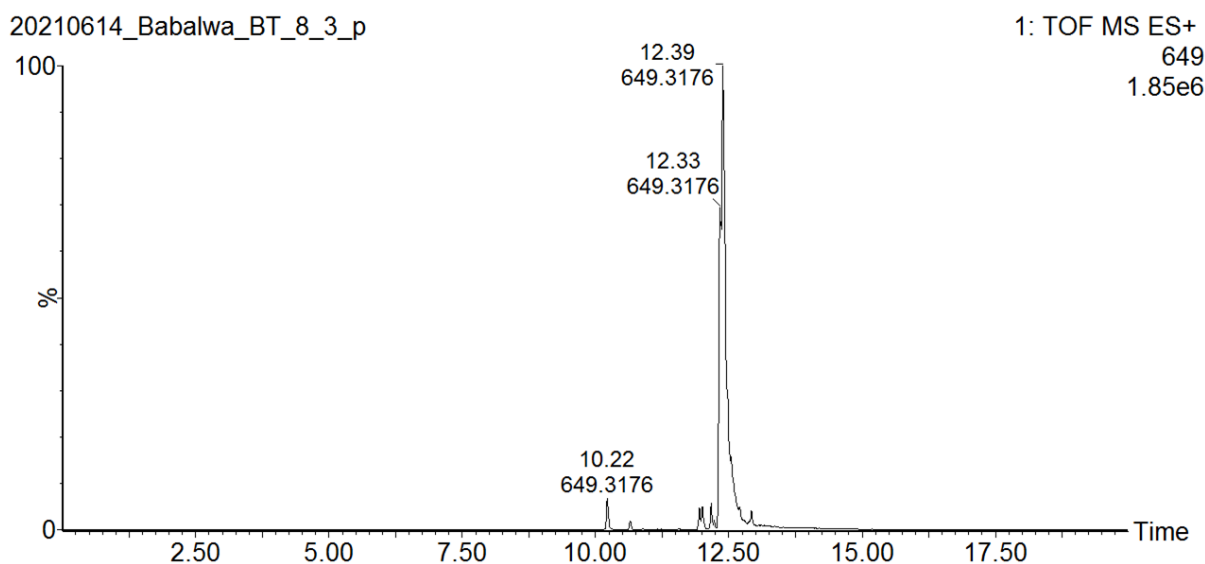
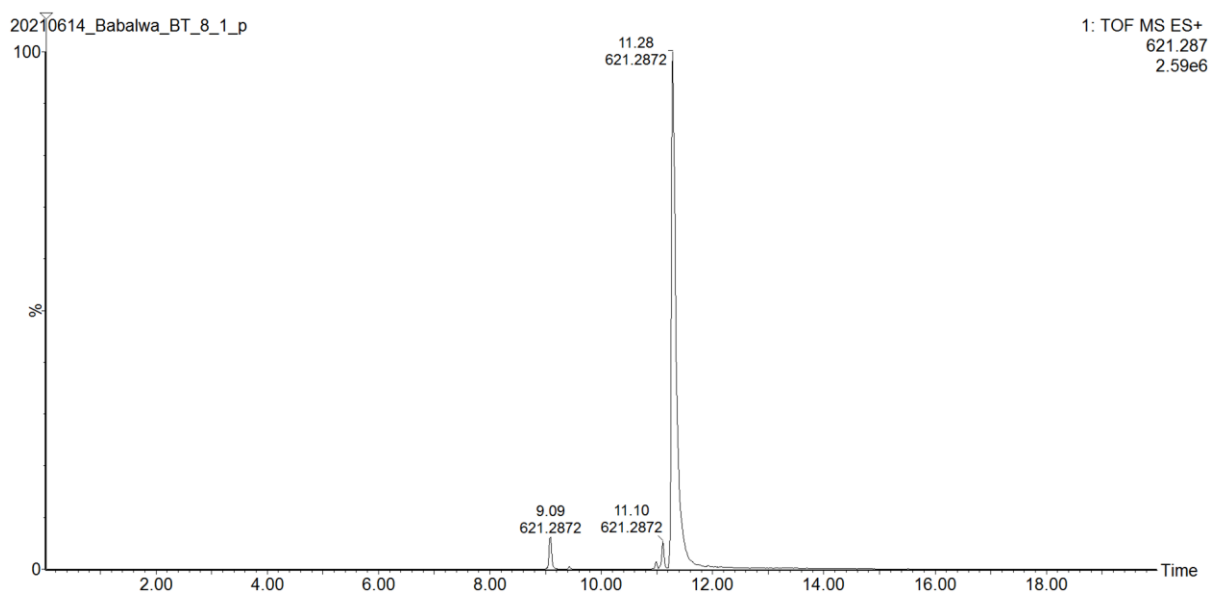


Figure 3. 21: The UPLC-QTOF-MS profile of isolated compounds tentatively identified as BT8-1 and BT8-3 in ESI positive mode isolated from the active fraction BT22-11/6.

Similarly, the active fraction BT-8 was further purified using a mass-directed purification approach on a preparative HPLC. The two peaks in the ESI positive mode (Figure 3.22) corresponding to the daphnane diterpenoids with similar m/z values of 647.3044 were targeted and collected in pure form. The compounds were labelled as BT8-2A (1.7 mg) and BT8-2B (0.93 mg). The ESI positive mode BPI chromatograms of the pure compounds BT8-2A and BT8-2B is shown in Figure 3.20.

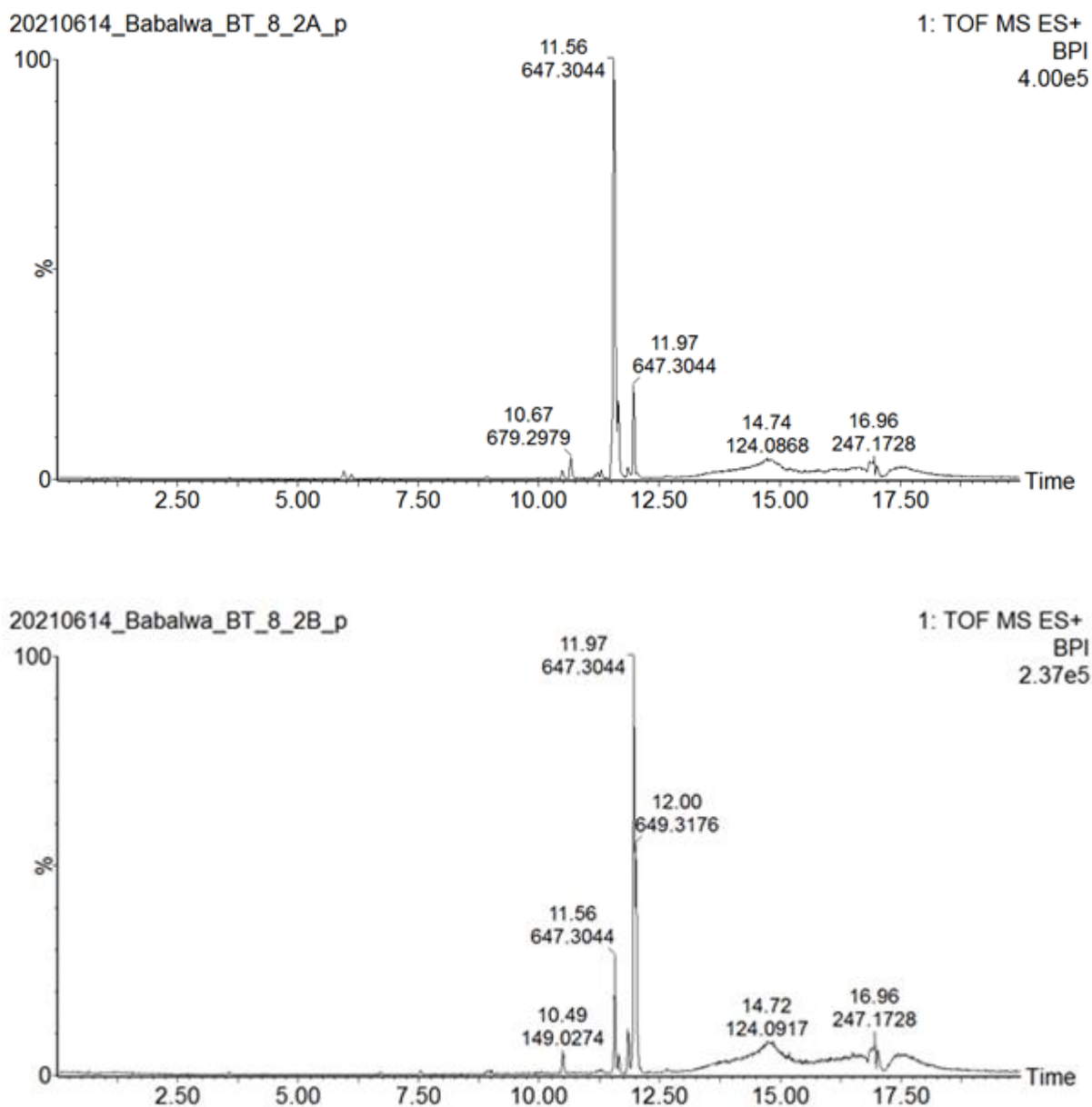


Figure 3. 22: The ESI positive mode of BPI chromatograms of pure compounds BT8-2A and BT8-2B isolated from the active fraction BT8.

Lastly, the preparative HPLC-MS daphnane rich fraction BT-GH-1 (350 mg) was further purified using a mass-directed purification approach on a preparative HPLC. The two peaks in the ESI positive mode (Figure 3.23) corresponded to the daphnane diterpenoids with similar m/z values of 649.2893 were targeted and collected in pure form. The compounds were labelled as BT-GH-5A (1.5 mg) and BT-GH-5B (4.00 mg). The ESI positive mode BPI chromatograms of the pure compounds BT-GH-5A and BT-GH-5B is shown in Figure 3.23.

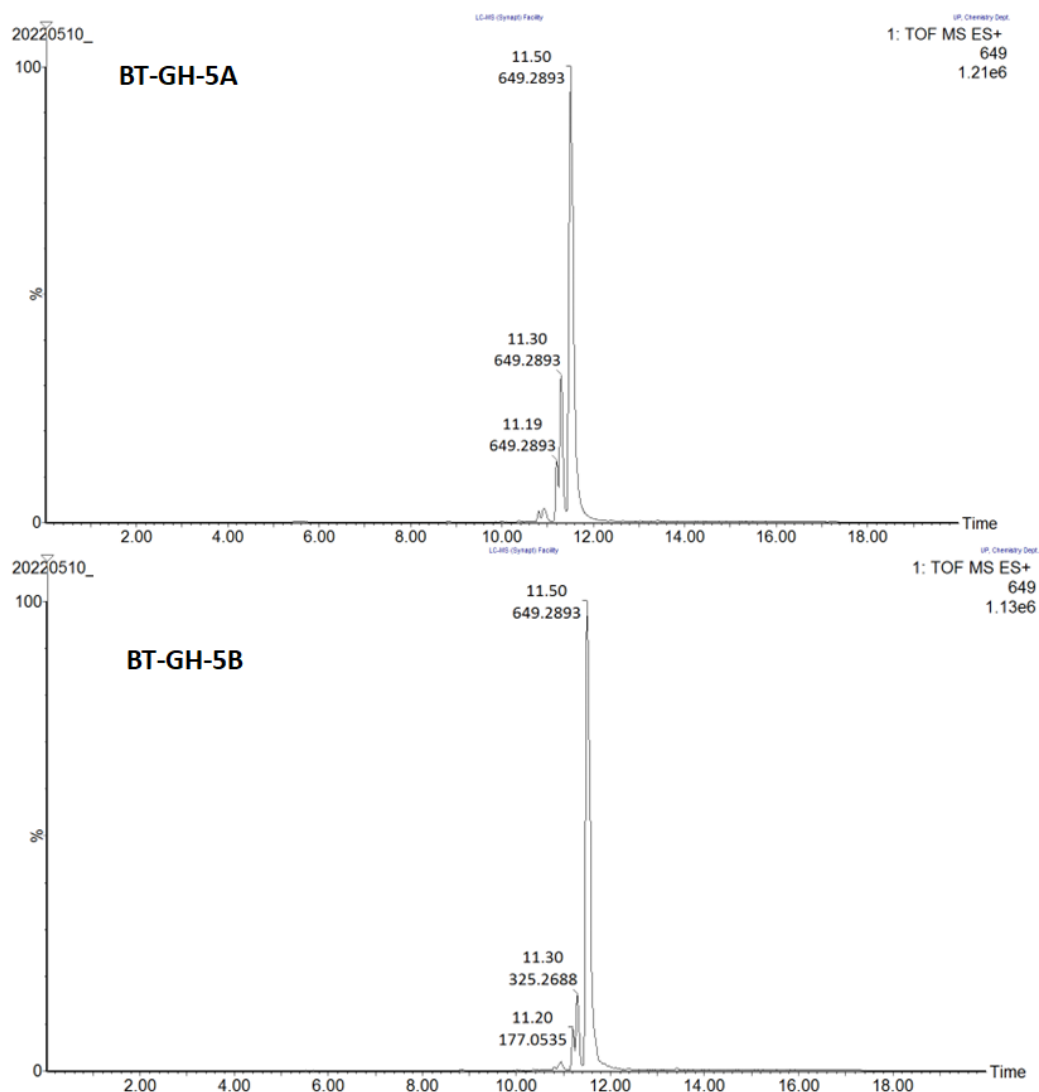


Figure 3. 23: The ESI positive mode of BPI chromatograms of pure compounds BT-GH-5A and BT-GH-5B isolated from the preparative HPLC-MS fraction BT-GH-1

3.7.13. Structure elucidation of compounds using NMR

3.7.13.1. Structure elucidation of lasiocephalin a chemical marker coumarin in the DCM extract

Lasiocephalin was obtained as the major compound in the extract, shown by a ratio of 2:1 in ^1H NMR spectra (Supplementary data 7) in the mixture of lasiocephalin : lasioerin. The compound was obtained as a white amorphous powder from the dichloromethane extract of *G. sericocephala* which will be used as a chemical marker for quality control purposes. The ESI positive mode on the QTOF mass spectrometer gave a precursor ion $[\text{M}+\text{H}]^+$ at m/z

337.0710 corresponding to molecular formula $C_{19}H_{12}O_6$ (calculated for $C_{19}H_{12}O_6$ 336.0830). The MS/MS spectrum of the compound revealed an intense ion at m/z 321.0547 due to a loss of a methyl group. A characteristic fragmentation pattern of a biscoumarin was depicted by the loss of two CO_2 was observed by presence of fragment ion at m/z 265.0632.^[10] The number of protons were confirmed by ^1H NMR and the number of carbon atoms was confirmed using ^{13}C NMR spectroscopy (shown in supplementary data 8). The structure of lasiocephalin is shown in Figure 3.24

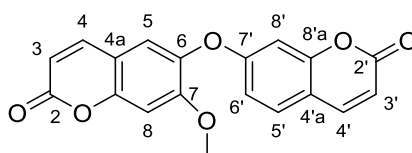


Figure 3.24: The Structure of lasiocephalin

The ^1H NMR spectrum revealed presence of twelve proton signals including one methoxy group at δ 3.89 (7-OMe). The presence of four distinct doublets at δ 6.37 (H -3'), δ 6.33 (1H, d, J = 9.5 Hz, H -3), δ 7.63 (1H, d, J = 9.6 Hz, H -4) and δ 7.69 (1H, d, J = 9.7 Hz, H -4') corresponding to two units of coumarins suggesting an unsymmetrical coumarin dimer. The ^1H NMR also displays two signals at δ 7.00 (1H, s, H -8) and δ 7.29 (1H, s, H -5) characteristic of aromatic protons of a 1, 2, 4, 5 tetra-substituted benzene ring as well as signals of aromatic protons of a 1, 3, 4 tri-substituted benzene ring at δ 6.77 (1H, d, J = 2.4 Hz, C-8'), δ 6.90 (1H, dd, J = 2.4, 8.5, C-6') and δ 7.44 (1H, d, J = 8.6 Hz, C-5').

The ^{13}C NMR spectrum confirmed nineteen carbon signals including one methoxy group δ 56.49, two carbonyl groups at δ 160.7 (C-2) and δ 160.8 (C-2'), as well as five oxygenated carbon signals at δ 161.2 (C-7'), δ 103.7 (C-8a'), δ 155.0 (C-7), δ 153.1 (C-8a) and δ 139.8 (C-6). The DEPT-135 and ^{13}C NMR spectrum confirmed the presence of nine quaternary carbons at δ 160.7 (C-2), δ 112.1 (C-4a), δ 139.8 (C-6), δ 155.0 (C-7), δ 153.1 (C-8a), δ 160.8 (C-2'), δ 114.1 (C-4a'), δ 161.2 (C-7') and δ 155.6 (C-8a'); nine CH carbons at δ 143.1 (C-4'),

δ 142.7 (C-4), δ 129.1 (C-5'), δ 120.4 (C-5), δ 114.4 (C-3), δ 114.2 (C-3'), δ 113.2 (C-3), δ 103.7 (C-8') and δ 101.3 (C-8); one methyl group (methoxy) at δ 56.5 (7-OMe).

The HSQC and COSY correlations (supplementary data 9) led to the assignments of the proton and carbon signals as shown in Table 3.2 while the ^1H and ^{13}C NMR data matched the published data by Tanemossu *et al.*, (2014).^[10]

Table 3. 2: Comparison of ^1H and ^{13}C chemical shifts of isolated lasiocephalin recorded using a 500 MHz NMR with published data in CDCl_3

Position	Lasiocephalin CDCl_3 (^1H , 500 MHz ^{13}C , 125 MHz)		Published data for lasiocephalin in CDCl_3 (^1H , 600 MHz ^{13}C , 150 MHz) ^[3]	
	δ (ppm)	δ (ppm), J in Hz	δ (ppm)	δ (ppm), J in Hz
2	160.7 C		160.6	-
3	114.4 CH	6.37 (d, 9.5)	114.4	6.35 (d, 9.2)
4	142.7 CH	7.63 (d, 9.5)	142.6	7.60 (d, 9.5)
4a	112.1 C	-	112.1	-
5	120.4 CH	7.29 (s)	120.3	-
6	139.8 C	-	139.8	-
7	155.0 C	-	154.9	-
8	101.3 CH	7.00 (s)	101.3	7.22 (s)
8a	153.1 C	-	153.1	-
2'	160.8 C	-	160.7	-
3'	114.2 CH	6.33 (d, 9.5)	114.3	6.30 (d, 9.5)
4'	143.1 CH	7.69 (d, 9.7)	143.0	7.66 (d, 9.5)
4a'	114.1 C	-	114.0	-
5'	129.1 CH	7.44 (d, 8.6)	129.0	7.57 (d, 8.8)
6'	113.2 CH	6.90 (dd, 2.4, 8.5)	113.1	6.88 (dd, 2.6, 8.8)
7'	161.2 C	-	161.2	-
8'	103.7 CH	6.77 (d, 2.4)	103.8	6.76 (d, 2.6)
8a'	155.6 C	-	155.6	-
7-OMe	56.5 CH_3	3.89 (s)	56.6	3.89 (s)

The HMBC correlations were used to establish the attachment of the methoxy group and to determine the linkage between the two coumarins. The HMBC spectrum gave a strong correlation between protons δ 7.29 (C-5) with δ 142.7 (C-4), δ 101.3 (C-8), δ 155.6 (C-8'a), δ 153.1 (C-8a), and δ 155.0 (C-7) confirming the position oxygen bridge linking the two coumarins at C-7. The proton at δ 3.89 gave a strong correlation with δ 155.0 (C-7) and confirmed the attachment of a methoxy group to be at C-7 (Supplementary data 9). The COSY correlation between δ 6.37 (H-3) and δ 7.63 (H-4); δ 7.44 (H-5') and 6.90 (H-6'); δ 6.33 (H-3') and δ 7.69 (H-4') allowed for the assignment of the protons in the compound (supplementary data 10). The COSY and HMBC correlations are shown in Figure 3.25.

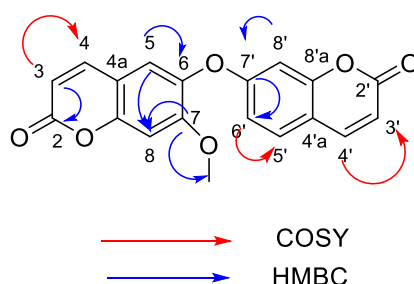


Figure 3. 25: COSY and HMBC correlations in lasiocephalin

Lasiocephalin is a biscoumarin originally isolated from *Lasiosiphon eriocephalus* of the *Thymelaeaceae* family a traditional medicinal plant of Europe and Northern America.^[10] The plant has been reported to have anti-coagulant activity.^[23] The compound has also been isolated from *Hypericum riparium* (*Guttiferae*).^[25] This study presents for the first time the isolation of lasiocephalin in *G. sericocephala*.

3.7.13.2. Structure elucidation of Lasioerin a chemical marker of the DCM extract of *G. sericocephala*

Lasioerin a known biscoumarin was isolated as a white amorphous powder, the ESI positive mode on the QTOF mass spectrometer gave a precursor ion $[M+H]^+$ at m/z 305.0439 corresponding to molecular formula $C_{18}H_9O_5$ (calculated for $C_{18}H_8O_6$ 304.0837 (Supplementary data 11). The pure compound was obtained in insufficient quantities and thus its chemical structure was elucidated using the NMR spectrum of the mixture with lasiocephalin present. The structure of lasioerin is shown in Figure 3.26

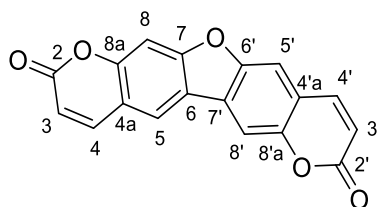


Figure 3. 26: The structure of lasioerin

The ^1H NMR spectrum of compound 2 showed a presence of four aromatic signals with chemical shifts δ 7.87 (1H, d, J = 9.5, H-4), δ 7.57 (1H, d, J = 8.6, H-5), δ 7.66 (1H, d, J = 8.4, H-8), and δ 6.55 (1H, d, J = 9.6, H-3). The doublets seen at δ 7.66 and δ 7.57 were due to the coupling between protons present in lasiocephalin as the signals are the singlets for lasioerin.

The ^{13}C spectrum revealed a presence of ester moiety with a chemical shift of δ 159.88 (C-2), the presence of four CH bonds at δ 143.7 (C-4), δ 128.0 (C-5), δ 115.3 (C-3) and δ 108.6 (C-8). The DEPT-135 with ^{13}C NMR confirmed the presence of five quaternary carbons at δ 159.9 (C-2), δ 158.4 (C-7), δ 149.4 (C-8a), δ 114.8 (C-4a) and δ 112.2 (C-6).

The HSQC and COSY correlations (supplementary data 12) led to the assignments of the ^1H and ^{13}C spectra signals as shown in Table 3.3.

Table 3.3: ^1H and ^{13}C NMR spectrum of lasioerin recorded in CDCl_3 using a 500 MHz NMR

Position	^{13}C (ppm)	^1H (ppm), J in Hz	COSY	HMBC
2	159.9 C	-	-	
3	115.3 CH	6.55 (d, 9.6)	H-4	C-4, C-2, C-4a
4	143.0 CH	7.87 (d, 9.5)	H-3	C-5, C-8a, C-2, C-6
4a	114.8 C	-	-	
5	128.0 CH	7.57 (d, 8.6)	-	C-6, C-4a, C-8a, C-7
6	112.2 C	-	-	
7	158.4 C	-	-	
8	108.6 CH	7.66 (d, 8.4)	-	C-6, C-4, C-8a, C-7
8a	149.4 C	-	-	

The ^1H - ^1H COSY spectra showed a correlation between δ 6.55 (C-3) and δ 7.87 (C-4) and allowed for the assignment of the protons (Supplementary data 13). The HMBC correlations were used to establish the attachment of the oxygen bridge and the linkage between the two coumarins (Supplementary data 3.14). The strong correlation between δ 7.66 (H-8) and δ 159.9 (C-7) and the downfield chemical shift of C-7 suggested that the ether bridge was connected from C-7. The upfield chemical shift of δ 112.2 (C-6) also indicated that C-6 is linking the two coumarins by a carbon-carbon bond. The COSY and HMBC correlations are shown in Figure 3.27.

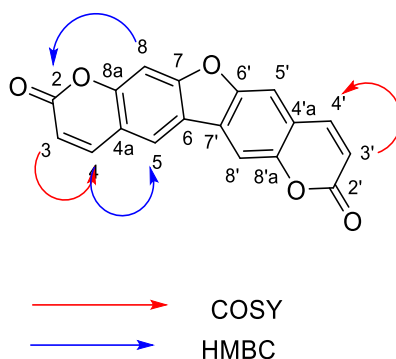


Figure 3. 27: COSY and HMBC correlations

Lasioerin is a biscoumarin originally isolated from *Lasiosiphon eriocephalus* of the *Thymelaeaceae* family a traditional medicinal plant of Europe and Northern America.^[10] The plant has been reported to have anti-coagulant activity.^[23] This study presents for the first time the isolation of lasioerin in *G. sericocephala*.

3.7.13.3. Structure elucidation of yuanhuacine A

The pure compound isolated from fraction BT-8 was identified as yuanhuacine A, known daphnane diterpenoid based on its NMR data, mass spectral data and comparison with published data (Supplementary data 15).^[3] The compound was obtained as a yellow amorphous powder, the ESI positive mode on the QTOF mass spectrometer gave a precursor ion $[M+H]^+$ at m/z 621.0360 corresponding to molecular formula $C_{35}H_{41}O_{10}$ (calculated for $C_{35}H_{40}O_{10}$ 620.0360).

The ^1H spectrum revealed 40 proton signals and ^{13}C revealed the presence of 35 carbon atoms, this corresponded with data confirmed with QTOF-MS. The structure of yuanhuacine A is shown in Figure 3.28.

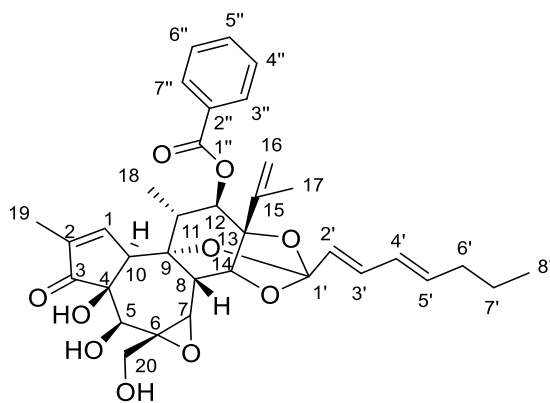


Figure 3. 28: The structure of yuanhuacine A

The ^1H NMR (CDCl_3 , 500 MHz, (Supplementary data 16) spectrum showed the presence of four methyl groups at δ 1.89 (3H, br s, C-17), δ 1.79 (3H, dd, $J = 1.3$, C-19), δ 1.41 (3H, d, $J = 7.7$, C-18) and δ 0.93 (3H, t, $J = 7.3$, C-8'). The characteristic presence of an isopropenyl moiety at δ 1.89 (H-17) and δ 5.03; 5.50 (H-16). The presence of a substituted phenyl group was indicated by signals at δ 7.92 (2H, dd, 8.2, 8.4, C-3'' and C-7''), δ 7.48 (2H, t, 8.1, 15.2, C-4'' and C-6'') and δ 7.61 (1H, m, C-5'').

The ^{13}C NMR (Supplementary data 17) spectra further classified by DEPT indicated a presence of an α - β ketone at δ 209.5 (C-3), The signals at δ 117.1 C-1' is an indication of an orthoester moiety. The orthoester is formed between C9/C13/C14 at δ 80.5 (C-9), δ 83.9 (C-13) and δ 79.0 ppm (C-14). An indication of a carbonyl group at δ 165.5 (C-1'') and an upfield shift by signals at δ 60.6 (C-6) and δ 64.7 (C-7) suggested a 6-7 epoxy moiety. The effect of the α , β ketone gave the downfield chemical shift for C-19 at δ 9.90. The presence of three alcohol groups δ 64.1 (C-20) primary alcohol, δ 72.2 (C-5) secondary alcohol and δ 72.0 (C-4) tertiary alcohol were present. The DEPT confirmed the presence of 10 quaternary carbons with a carbon-oxygen bond, six olefinic carbons and five methylene group (Supplementary data 18).

The HSQC and COSY correlation led to the assignment of the proton and carbon signals. The chemical shifts for ^1H and ^{13}C compared to published data are shown in Table 3.4.

Table 3. 4: Comparison of ^1H and ^{13}C chemical shifts of isolated yuanhuacine A recorded using a 500 MHz NMR with published data in CDCl_3

Position	Yuanhuacine A in CDCl_3 (^1H , 500 MHz and ^{13}C , 125 MHz)		Published data for Yuanhuacine A in $\text{CDCl}_3\text{-}d_1$ (^1H , 500 MHz ^{13}C , 125 MHz) ^[3]	
	δ (ppm)	δ (ppm), J in Hz	δ (ppm)	δ (ppm), J in Hz
1	160.5, CH	7.48 (t, 8.1)	160.9, CH	7.57 (s)
2	137.0, C	-	136.8, C	-
3	209.5, C	-	210.2, C	-
4	72.0, C	-	72.3, C	-
5	72.2, CH	4.25 (s)	72.2, CH	4.21 (s)
6	60.6, C	-	61.4 C	-
7	64.7, CH	3.84 (d, 13.0)	64.5, CH	3.63 (s)
8	34.8, CH_2	3.65 (d, 2.6)	36.8, CH	3.63 (d, 2.4)
9	80.5, C	-	78.5, C	-
10	47.5, CH	3.87 (quint, 5.2)	48.0, CH	3.86 (quint, 2.5)
11	44.2, CH	2.58 (q, 7.3)	44.5, CH	2.56 (q, 7.0)
12	78.2, CH	5.24 (s)	79.5, CH	5.23 (br s)
13	83.9, C	-	84.6, C	-
14	79.0, CH	4.92 (d,1.2)	81.1, CH	4.90 (d, 2.4)
15	143.0, C	-	143.2, C	-
16	113.7, CH_2	5.02 (m) 5.03 (s)	114.0, CH_2	5.02 (br m) 4.99 (br m)
17	18.8, CH_3	1.89 (bs)	19.2, CH_3	1.87 (br s)
18	18.4, CH_3	1.41 (d, 7.7)	18.9, CH_3	1.39 (d, 7.0)
19	9.9, CH_3	1.79 (dd, 1.3)	10.3, CH_3	1.77 (br s)
20	64.1, CH_2	3.97 (d, 12.7) 3.67 (s)	65.0, CH_2	3.91 3.83 (ABq, 12.4)
1'	117.1, C	-	117.7, C	-
2'	122.3, CH	5.70 (d, 15.4)	122.9, CH	5.68 (d, 15.5)
3'	135.2, CH	6.72 (dd, 10.9, 15.3)	135.6, CH	6.70 (dd, 15.5, 10.5)

4'	128.6, CH	6.09 (dd 10.8, 15.3)	129.2, CH	6.07 (dd, 15.2, 10.5)
5'	139.2, CH	5.89 (dt, 15.3, 14.5)	139.4, CH	5.87 (dt, 15.2, 7.2)
6'	35.8, CH ₂	2.11 (q, 7.3)	35.2, CH ₂	2.01 (q, 7.2)
7'	22.2, CH ₂	1.44 (sext, 7.3)	22.8, CH ₂	1.33 (sex, 7.2)
8'	13.7, CH ₃	0.93 (t, 7.3)	14.2, CH ₃	0.91 (t, 7.2)
1''	165.5, C	-	166.0, C	-
2''	129.5, C	-	Not detected	
3''-7''	129.7, CH	7.92 (dd, 8.2, 8.4)	129.9, CH	7.89 (br d, 8.0)
4''-6''	128.8, CH	7.48 (t, 8.1, 15.2)	128.9, CH	7.44 (br t, 8.0)
5''	133.5, CH	7.61 (m)	133.6, CH	7.57 (t, 8.0)

Analysis of the ¹H NMR, ¹³C NMR, and HMBC spectra enabled the correlation of protons to their respective carbon atoms, and further confirmed that the compound was a daphnane diterpene ester. In the HMBC spectrum, the carbon signal at δ 117.1 (C-1') correlated with the proton signals at δ 4.92 (H-14), δ 5.70 (H-2'), and δ 6.72 (H-3'), confirming the presence of an orthoester group in the compound. The H-7 (δ 3.84) correlated with C-5 (δ 72.2), and C-6 (δ 60.6), H-17 (δ 1.89) correlated with C-13 (δ 83.9), C-16 (δ 113.7), and C-15 (δ 143.0) showed the linkage and oxygenated patterns of these carbons (Supplementary data 19). The H-¹H COSY spectrum showed the correlations between δ 5.70 (H-2'), δ 6.72, (H-3'), δ 6.09 (H-4'), and δ 5.873 (H-5'), which represented trans-conjugated-diene. The protons coupling at δ 7.551, δ 3.833, and δ 1.764 were assigned to H-1, H-10, and H-19, respectively.

¹H-¹H COSY spectrum showed coupling between δ 5.70 (1H, d, J = 15.4, H-2') and δ 6.72, (dd, J =10.9, 15.3, H-3'), δ 6.09 (H-4'), and δ 5.87 (H-5'), which represented trans-conjugated-diene due to the large coupling constant and suggested that the alkyl group was a straight chain. The relative stereochemistry was deduced based on ¹H-¹H J coupling and on the basis of NOE analysis. A small coupling constant of 2.6 Hz was observed on H-8, this value is similar to previously reported values for this proton. [28] The NOE interactions observed between H-12 and CH₃-18 suggested that the protons are facing the same side. Additionally, the NOE

observed between H-11 and the benzoyl functional group at C-12 suggested that H-12 and H-11 are facing opposite sides. Yuanhuacine A is a 6-epoxy daphnane diterpenoid, these types of diterpenoids have been proven to have a C-6 α epoxy structure and a C-5 β hydroxyl group in ring B of the structure, due to the already proven configuration on this carbon the functional group at C-5 was assigned a β orientation.^[28] A coupling constant of 5.2 Hz was observed for H-10, the NOE interaction between H-5 and H-10 suggested that the protons are axial-equatorial. The large coupling constant of more than 12 Hz observed between H-7 and CH₂-20 and the NOE interaction between the protons suggested that the protons are trans-diaxial to each another. The small coupling constant of not more than 3 Hz observed in proton H-8 (2.6 Hz) and H-14 (1.2 Hz) and the NOE interaction observed between the protons suggested that the protons are trans-diequatorial to each other. (Supplementary data 20). The HMBC, NOE and COSY correlations are shown in Figure 3.29.

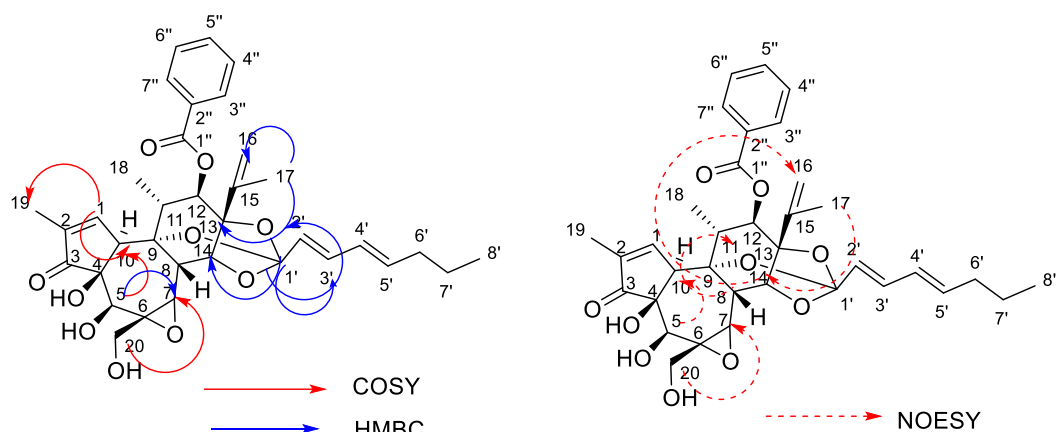


Figure 3. 29: COSY, NOESY and HMBC correlations in yuanhuacine A

Yuanhuacine A is a daphnane diterpenoid originally isolated from the roots of *Gnidia polycephala* a traditional plant endemic in Southern Africa is used to treat a variety of ailments by the Botswana people and has been reported to contribute to livestock poisoning due to its toxic compounds.^[3] This study reports the first isolation of yuanhuacine A in *G. sericocephala* roots.

3.7.13.4. Structure elucidation of yuanhuacine

The pure compound isolated from fraction BT-GH-5B was identified as yuanhuacine, a known daphnane diterpenoid based on its NMR data, mass spectral data and comparison with published data (Supplementary data 21).^[3] The compound was obtained as a white amorphous powder, the ESI positive mode on the QTOF mass spectrometer gave a precursor ion $[M+H]^+$ at m/z 649.2893 corresponding to molecular formula $C_{37}H_{45}O_{10}$ (calculated for $C_{37}H_{44}O_{10}$ 648.3096), (Supplementary 22). The ^1H spectrum revealed 44 proton signals and ^{13}C revealed the presence of 37 carbon atoms, this corresponded with data confirmed with QTOF-MS. The structure of yuanhuacine is shown in Figure 3.30.

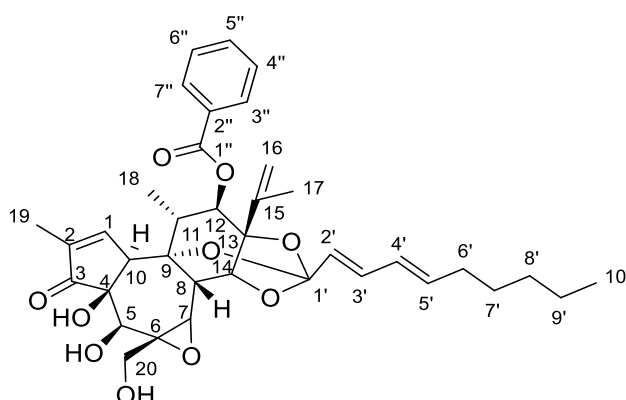


Figure 3.30: The structure of yuanhuacine

The ^1H spectra (Supplementary data 23) of the compound showed a presence four methyl groups at δ 1.86 (3H, s, H-17), δ 1.77 (3H, q, J = 2.6, H-19), δ 1.38 (3H, d, J = 7.3, H-18) and δ 0.88 (3H, t, J = 6.9, H-10'); a characteristic isopropenyl moiety at δ 1.86 (H-17) and δ 5.00 (H-16); The presence of a substituted phenyl group was indicated by signals at δ 7.89 (1H, dd, 7.1 C-3'' and C-7''), δ 7.48 (1H, dt, 7.8, 1.8, C-4'' and C-6'') and δ 7.59 (1H, m, C-5'').

The ^{13}C spectrum showed the presence of a characteristic orthoester moiety at δ 117.1 (C-1'); a carbonyl group at δ 129.5 (C-2''), presence of an α,β unsaturated ketone at δ 209.7 (C-3); the aromatic carbons represented by δ 133.4 (C-5''), δ 128.8 (C-4''/6'') and δ 129.5 (C-3''/7''). The presence of an epoxy moiety at δ 71.8 (C-7) and δ 72.1 (C-6). The spectrum also showed three hydroxy bearing carbons with chemical shifts δ 60.6 (C-4), δ 64.7 (C-5) and δ 63.4 (C-20); an isopropenyl moiety at carbons C-16 and C-17 with chemical shifts at δ 114.1 and δ 18.9 (Supplementary 3.23). The HSQC and COSY correlation led to the assignment of the proton and carbon signals. The chemical shifts for ^1H and ^{13}C compared to literature data are shown in Table 3.5.

Table 3. 5: Comparison of ^1H and ^{13}C chemical shifts of isolated yuanhuacine recorded using a 500 MHz with published data in CDCl_3

Position	Yuanhuacine in CDCl_3 (^1H , 500 MHz and ^{13}C , 125 MHz)		Published data for Yuanhuacine in CDCl_3 (^1H and ^{13}C , 400 MHz)	
	δC	δH	δC	δH
1	160.5, CH	7.59 (m)	160.5	7.55 (s)
2	137.1	-	137.1	-
3	209.8	-	209.6	-
4	60.8	-	60.8	-
5	64.0, CH	3.64, (s)	64.2	3.63 (s)
6	72.2	-	72.4	-
7	72.3, CH	4.22, (br. s)	71.9	4.19 (s)
8	35.7, CH	3.62, (d, 2.5)	35.9	3.63 (s)
9	78.2	-	78.4	-
10	47.6, CH	3.84 (m)	47.6	3.82 (m)
11	44.8, CH	2.55, (q, 6.6)	44.6	2.55 (d 7.2)
12	79.0, CH	5.21, (s)	79.1	5.22 (s)
13	83.9	-	84.0	-
14	80.7, CH	4.89, (d, 2.5)	80.6	4.90 (d)
15	142.9	-	143.2	-
16	113.8, CH_2	5.00, (d, 5.1)	113.8	5.02 (s)
17	18.8, CH_3	1.86, (s)	18.9	1.87 (s)

18	18.3, CH ₃	1.38, (d, 7.3)	18.5	1.39 (d, 7.2)
19	9.8, CH ₃	1.77, (q, 2.6)	10.0	1.76 (d, 7.2)
20	64.7, CH ₂	3.92, (m)	64.8	3.84 (s); 3.93 (s)
1'	117.1	-	117.2	-
2'	122.8, CH	5.67, (d, 15.7)	122.4	5.68 (d, 15.6)
3'	135.3, CH	6.69, (dd, 10.6, 15.5)	135.23	6.70 (dd, 15.6, 10.8)
4'	128.6, CH	6.06 (dd, 11.0, 15.3)	128.8	6.07 (dd, 10.8, 10.8)
5'	139.6, CH	5.86, (m, 7.6, 15.2)	139.6	5.87 (m, 5.0, 7.2)
6'	32.5, CH ₂	2.10, (q, 6.6)	32.8	2.11 (d)
7'	28.7, CH ₂	1.38, (d, 7.3)	28.9	1.39 (m)
8'	31.5, CH ₂	1.28, (m)	31.5	1.29 (m)
9'	22.8, CH ₂	1.28, (m)	22.4	1.26 (m)
10'	14.1, CH ₃	0.88, (t, 6.9)	14.2	0.89 (t)
1"	165.6	-	165.6	-
2"	129.7	-	129.9	-
3"-7"	129.5, CH	7.89, (dd, 8.1)	129.6	7.89 (d, 7.6)
4"-6"	128.7, CH	7.45, (t, 7.9, 15.3)	128.8	7.44 (t, 7.6)
5"	133.5, CH	7.59, (m)	133.5	7.58 (s)

Analysis of the ¹H NMR, ¹³C NMR, and HMBC spectra enabled the correlation of protons to their respective carbon atoms, and further confirmed that the compound was a daphnane diterpene ester. In the HMBC spectrum, the carbon signal at δ 117.1 (C-1') correlated with the proton signals at δ 4.89 (H-14), δ 5.67 (H-2'), and δ 6.69 (H-3'), confirming the presence of an orthoester group in the compound. The H-7 (δ 3.84) correlated with C-5 (δ 64.0), and C-6 (δ 72.2), H-17 (δ 1.86) correlated with C-13 (δ 83.9), C-16 (δ 113.8), and C-15 (δ 142.9) showed the linkage and oxygenated patterns of these carbons (Supplementary data 24).

The ¹H-¹H COSY spectrum showed an interaction between δ 5.67 (1H, d, J = 15.7, H-2') and δ 6.69, (dd, J =10.6, 15.5, H-3'), δ 6.06 (dd, J = 11.0, 15.3, H-4'), and δ 5.87 (m, H-5'), which represented trans-conjugated-diene due to the large coupling constant and suggested that the

alkyl group was a straight chain. The relative stereochemistry was deduced based on ^1H – ^1H coupling constant and on the basis of NOE analysis. A small coupling constant of 2.6 Hz was observed on H-8, this value is similar to previously reported values for this proton. [28] The small coupling constants observed for protons at H-14 ($J = 2.5$ Hz) and the ^1H – ^1H coupling between H-8 and H-14 suggested that the protons are trans-diequatorial. The observed NOE interaction between H-8, H-11 and H-14 proved that the protons at these positions were facing the same side of the plane. No coupling was observed between H-11 and H-12 indicating that these two protons are in all likelihood at a 90° confirmation to each other. The large coupling constants observed H-3' and H-4' of 15.3 Hz resulted in the protons at C-3' and C-4' to be assigned a trans configuration. (Supplementary data 25). The HMBC, NOE and COSY correlations are shown in Figure 3.31.

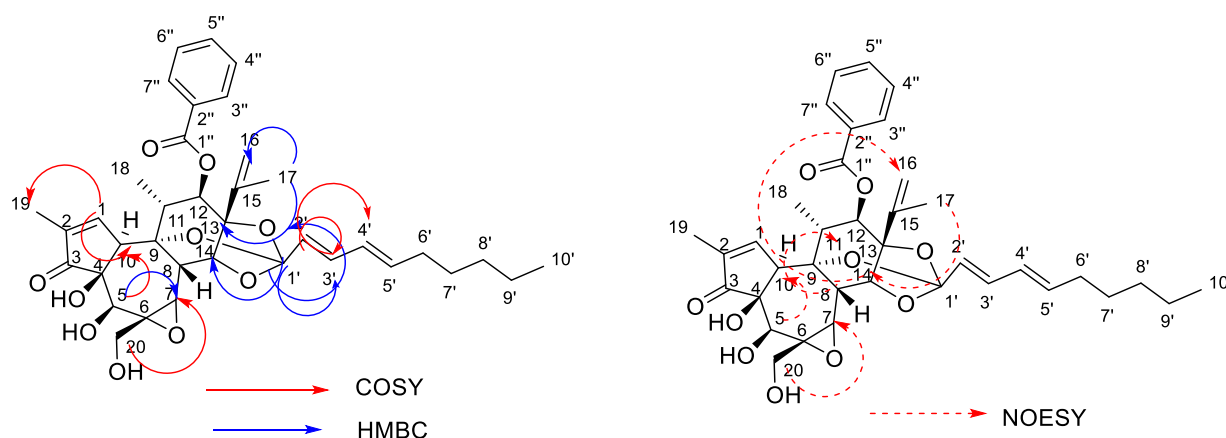


Figure 3. 31: COSY, NOESY and HMBC correlations in yuanhuacine

Yuanhuacine is a daphnane diterpenoid originally isolated from the roots of *Daphne genkwa* a traditional Chinese indigenous plant. The compound has been reported for its anti-viral and anti-cancer properties amongst other pharmacological applications.[3] However, this is the first report to present the isolation of this compound from *G. sericocephala* roots.

3.7.13.5. Structure elucidation of cis 2''/3'' isomer of gnididin

The pure compound isolated from fraction BT-GH-5B was identified as a new daphnane diterpenoid based on its NMR data and mass spectral data (Supplementary data 26).^[3] The compound was obtained as a yellow amorphous powder, the ESI positive mode on the QTOF mass spectrometer gave a precursor ion $[M+H]^+$ at m/z 649.2893 corresponding to molecular formula $C_{37}H_{45}O_{10}$ (calculated for $C_{37}H_{44}O_{10}$ 648.3096), (Supplementary 27). The ^1H spectrum revealed 44 proton signals and ^{13}C revealed the presence of 37 carbon atoms, this corresponded with data confirmed with QTOF-MS elemental analysis.

The ^1H spectra (Supplementary data 28) of the compound showed a presence four methyl groups at δ 1.87 (3H, s, H-17), δ 1.78 (3H, q, $J = 13.8$, H-19), δ 1.38 (3H, m, H-18) and δ 0.87 (3H, t, $J = 6.5$, H-10''), a characteristic isopropenyl moiety at δ 1.87 (3H, s, H-17) and δ 5.00 (2H, d, $J = 3.1$, H-16); The presence of a substituted phenyl group was indicated by signals at δ 7.72 (1H, m, C-3' and C-7'), δ 7.38 (1H, m, C-4' and C-6') and δ 7.38 (1H, m, C-5'). The ^1H -NMR data of the compound showed a cis geometry for H-2'' and H-3'' due to a coupling constant of 11.5 Hz.^[27] Additionally, the ^1H -NMR data of the compound showed a trans geometry at H-5'' due to the large coupling constant of 14.9 Hz.^[27] The ^1H -NMR data of the compound was relatively similar to that of gnididin a trans/trans diene in its alkyl chain at positions H-2'' and H-3'' and H-4'' and H-5''.^[27] The structure of the cis- 2''/3'' isomer of gnididin is shown in Figure 3.32.

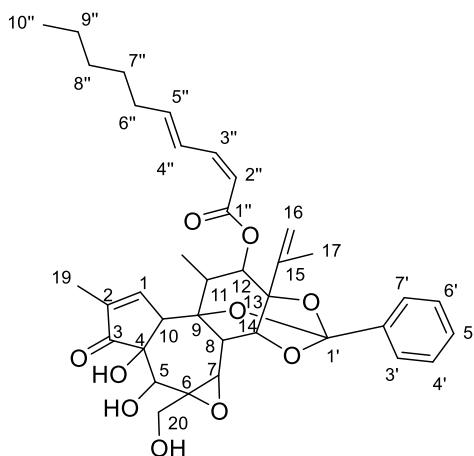


Figure 3. 32: The cis-2''/3'' isomer of gnididin

The ^{13}C spectrum showed the presence of a characteristic orthoester moiety at δ 117.1 (C-1'); a carbonyl group at δ 165.5 (C-1''), presence of an α,β unsaturated ketone at δ 209.8 (C-3); the aromatic carbons represented by δ 129.7 (C-5'), δ 128.7 (C-4'/6') and δ 126.0 (C-3'/7'). The presence of an epoxy moiety at δ 72.3 (C-7) and δ 72.2 (C-6). The spectrum also showed three hydroxy bearing carbons with chemical shifts δ 60.4 (C-4), δ 64.5 (C-5) and δ 64.4 (C-20); an isopropenyl moiety at carbons C-16 and C-17 with chemical shifts at δ 114.2 and δ 18.4 (Supplementary 29). The HSQC and COSY correlation led to the assignment of the proton and carbon signals. The chemical shifts for ^1H and ^{13}C are shown in Table 3.6.

Table 3. 6: ^1H and ^{13}C chemical shifts of isolated cis- 2''/3'' isomer of gnididin recorded using a 500 MHz NMR with published data in CDCl_3

Position	δ_{C} (ppm)	δ_{H} (ppm) J in Hz	COSY ($\text{H}^1\text{-H}^1$)	HMBC
1	160.7 CH	7.59 (q, 1.5,2.4)	H-10	C-10
2	137.0 C	-		
3	209.8 C	-		
4	60.4 C	-		
5	64.5 CH	3.61 (s)		
6	72.2 C	-		
7	72.3 CH	4.27 (s)	H-8	C-4; C-5; C-6
8	35.5 CH	3.61 (s)	H-7	C-10; C-12,

9	80.9 C	-		
10	47.5 CH	3.92 (m)		
11	44.2 CH	2.55 (d,)		
12	77.9 CH	5.09 (s)		C-12; C-17; C-13, C-15, C-1"
13	84.3 C	-		
14	80.9 CH	4.90 (d, 2.7)		C-15; C-17
15	142.8 C	-		
16	114.2 CH ₂	5.02 (d, 3.1)	H-16	C-13; C-15
17	19.0 CH ₃	1.87 (s)	H-17	
18	18.4 CH ₃	1.38 (m)		
19	9.9 CH ₃	1.78 (q, 13.8)		C-1; C-2; C-3
20	64.4 CH ₂	3.92 (m)		
1'	117.8 C	-		C-14; C-3'/C-7'
2'	137.0 C	-		
3'/7'	126.0 CH	7.72 (m)	H-4'/H-6'	C-1'; C-5'
4'/6'	128.7 CH	7.38 (m)	H-3'/H-7'	C-2'; C-3'/C-7'
5'	129.7	7.38 (m)		C-2'; C-3'/C-7'
1"	165.5 C	-		C-5"; C-3"
2"	114.2 CH	5.50 (d, 11.5)	H-3"	
3"	147.2 CH	6.57 (t, 11.5)	H-2"; H-4"	C-5"; C-1"
4"	126.8 CH	7.37 (m)	H-3"; H-5";	C-2";
5"	147.4 CH	6.09 (quint, 7.4, 14.9)	H-6"	C-3";
6"	28.5 CH ₂	2.18 (q)	H-5"	
7"	32.8 CH ₂	2.35 (m)		
8"	35.5 CH ₂	3.64 (d,2.6)		
9"	22.5 CH ₂	1.28 (m)	H-9"	
10"	14.0 CH ₃	0.87 (t, 6.5)	H-10"	

Analysis of the ¹H NMR, ¹³C NMR, and HMBC spectra enabled the correlation of protons to their respective carbon atoms, and further confirmed that the compound was a daphnane diterpene ester. In the HMBC spectrum, the carbon signal at δ 117. 8 (C-1') correlated with

the proton signals at δ 7.38 (1H, m, H-3'/ H-7') and δ 4.90 (1H, d, 2.7, H-14), confirming the linkage of an orthoester moiety to the phenyl group. The attachment of the alkyl chain was determined through the correlation between δ 147.2 (C-1'') and δ 6.57 (1H, t, J = 11.5, H-3''); δ 6.09 (1H, quint, J = 7.9, 14.9, H-5'') (Supplementary data 30).

The ^1H – ^1H COSY spectrum showed an interaction between δ 4.27 (1H, s, H-7) and δ 6.69, (1H, s, H-8 the interactions observed between δ 7.72 (1H, m, H-4'/ H-6') and δ 7.38 (1H, m, H-3'/ H-7') allowed for the assignment of the protons in the aromatic region. The ^1H – ^1H COSY interactions observed between δ 6.57 (1H, t, J = 11.5, H-3''); δ 5.50 (1H, d, J = 11.5, H-2'') and δ 7.37 (1H, m, H-4''), allowed for the assignment of these protons in the alkyl chain. The coupling constant J = 11.5 Hz between H-2'' and H-3'' suggested a cis geometry, while the observed J = , 14.9 at H-5'' suggested that the proton had a trans geometry. The relative stereochemistry was deduced based on ^1H – ^1H coupling constant and on the basis of NOE analysis (Supplementary data 31). A small ^1H – ^1H coupling constant of 2.7 Hz observed for H-14 and the NOE interaction between H-14 and H-8 suggested that the protons are trans-diequatorial to each other. A small ^1H – ^1H coupling constant of not more than 2.4 Hz was observed for H-1, additionally the NOE interaction between H-1 and H-10 suggested that the protons were trans-diequatorial to each other. Consequently, the NOE interaction between H-5 and H-10 suggested that these protons are facing the same face of the ring. The NOE interaction observed between H-7 and CH₂-20 suggested that the protons are on the same side of the plane. The absolute configuration of the compound could not be determined due to insufficient quantities of the sample. The HMBC, NOE and COSY correlations are shown in Figure 3.33.

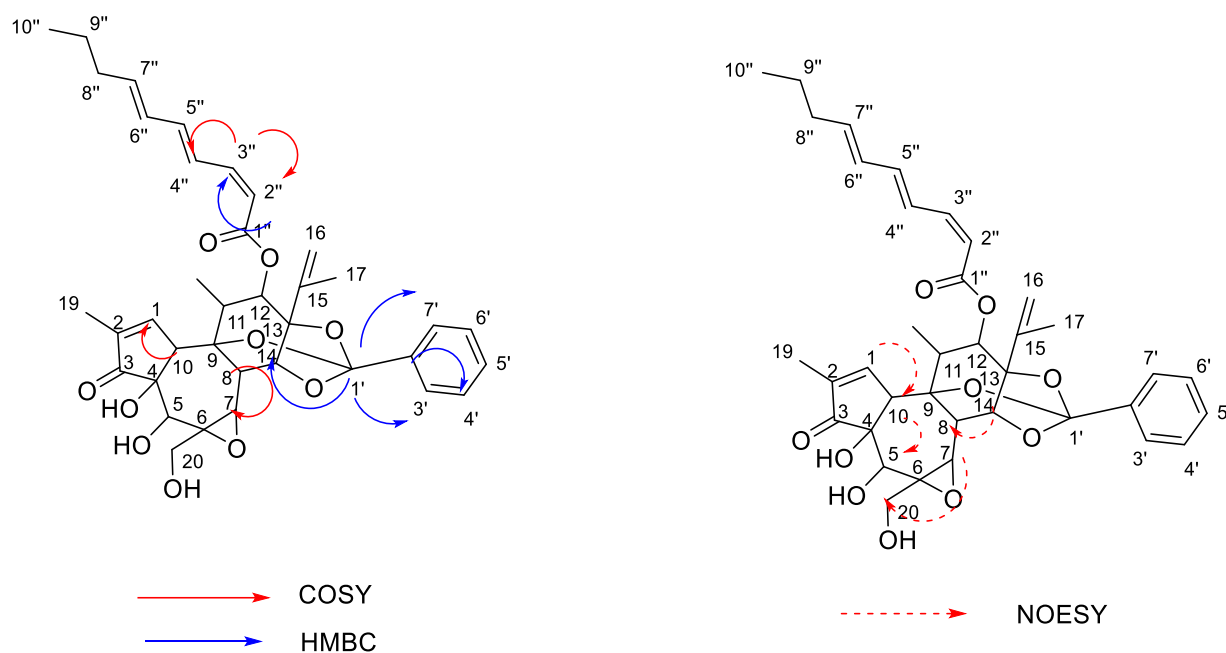


Figure 3. 33: COSY, NOESY and HMBC correlations in cis 2''/3'' isomer of gnididin

The cis 2''/3'' isomer of gnididin is reported for the first time in this study. These results also suggest that *G. sericocephala* can be used as a source of new drug leads with anti-HIV and latency reversal activity. Additionally, there is no available ^{13}C and ^1H NMR data available for gnididin, even though its long been reported to have a trans diene in its alkyl chain in numerous studies. The data obtained in this study could be an important source of reference for gnididin NMR spectral data.

3.7.13.6. Structure elucidation of yuanhuajine

The pure compound yuanhuajine (1.7 mg) was purified as a white amorphous compound. The ESI positive mode on the QTOF mass spectrometer gave a precursor ion $[\text{M}+\text{H}]^+$ at m/z 647.2858 corresponding to molecular formula $\text{C}_{37}\text{H}_{43}\text{O}_{10}$ (calculated for $\text{C}_{37}\text{H}_{42}\text{O}_{10}$ 646.2856) with 17 degrees of unsaturation (Supplementary 31). The ^1H -NMR spectroscopy revealed 42 proton signals and ^{13}C revealed the presence of 37 carbon atoms, this corresponded with data confirmed with QTOF-MS. The structure of yuanhuajine is shown in Figure 3.34.

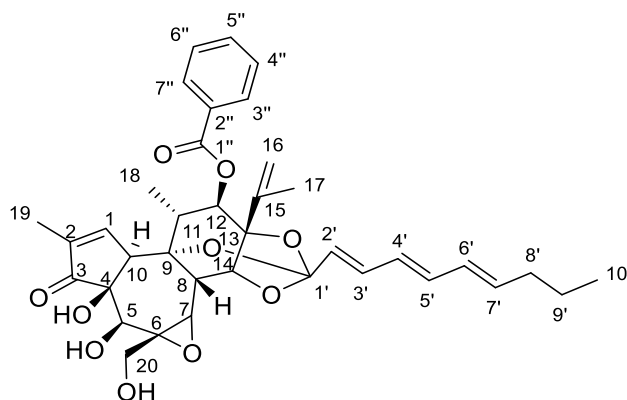


Figure 3. 34: Structure of yuanhuajine

The ^1H - NMR spectrum revealed the presence of four methyl groups at resonance signal δ 1.88 (3H, s, H-17), δ 1.75 (3H, s, H-19), δ 1.41 (3H, t, $J = 3.9$ Hz, H-18) and δ 0.92 (3H, t, $J = 7.4$, H-10'). The spectrum also showed an aromatic system showed by signals at δ 7.72 (2H, m, H-3''-H-7''), δ 7.59 (1H, m, H-5'') and δ 7.39 (2H, m, H-4''-H-6''), two aliphatic methylene protons at δ 2.1 (2H, q, 8.7, 15.8 H-8'), δ 1.44 (2H, m, H-9'). The characteristic presence of an isopropenyl moiety at δ 1.88 (H-17) and δ 5.02 (H-16) (Supplementary data 32).

The ^{13}C NMR spectra revealed the presence of an ortho-ester moiety at resonance signals δ 119.3 (C-1') joined at C9/13/14, a characteristic of daphnane diterpenoids. The ^{13}C signal at δ 209.50 (C-3) confirmed the presence of an α,β unsaturated carbonyl group a characteristic of daphnane diterpenoids. Signals at δ 135.6 (C-2'), δ 126.3 (C-3'/C-7'), δ 128.2 (C-4'/C-6') and δ 129.9 (C-5') confirmed the presence of an aromatic system. An aliphatic conjugated system was confirmed by signals at δ 135.3 (C-2''), δ 128.2 (C-3''), δ 127.2 (C-4''), δ 129.9 (C-5''), δ 127.2 (C-6'') and δ 141.5 (C-7'') (Supplementary data 33). The compound was confirmed as yuanhuajine by comparison of the ^{13}C and ^1H NMR spectra of the compound with literature data. The NMR backbone of yuanhuajine resembles that of yuanhuacine A with an additional $\text{CH}=\text{CH}$ with resonances at δ 6.16 (H- 5') and δ 5.97(H-6') that corresponded to δ 129.9 (C-5') and δ 141.5 (C-6'), respectively.

The HSQC and COSY correlation led to the assignment of the proton and carbon signals. The chemical shifts for ^1H and ^{13}C compared to literature data are shown in Table 3.6.

Table 3. 7: Comparison of ^1H and ^{13}C chemical shifts of isolated yuanhuajine recorded using a 500 MHz NMR with published data in CDCl_3

Position	Yuanhuajine in CDCl_3-d_1 (^1H , 500 MHz and ^{13}C , 125 MHz)		Published data for yuanhuajine in CDCl_3-d_1 (^1H , 500 MHz, ^{13}C , 125 MHz) ^[26,27]	
	δ (ppm)	δ (ppm), J in Hz	δ (ppm)	δ (ppm), J in Hz
1	160.1 CH	7.39 (d, 4.5)	160.5	7.57 (s)
2	137.0 C	-	137.1	
3	209.5 C	-	209.6	
4	78.4 C	-	60.8	
5	72.1 CH	4.27 (s)	64.2	3.65 (s)
6	60.5 C	-	72.4	
7	64.4 CH	3.98 (br d, 7.6, 4.7)	72.1	4.23 (s)
8	35.6 CH	3.64 (q, 12.4, 12.7)	36.0	3.63 (d)
9	72.2 C	-	78.5	
10	47.5 CH	3.93 (m)	47.7	3.85 (m)
11	44.2 CH	2.56 (m)	44.3	2.38 (d, 7.2)
12	78.6 CH	5.11 (t, 7.9)	79.2	5.22 (s)
13	84.3 C	-	84.2	
14	80.9 CH	4.93 (d, 2.5)	80.7	4.90 (d)
15	142.9 C	-	143.2	
16	113.8 CH_2	5.02 (d, 4.9)	113.8	5.01 (s)
17	1847 CH_3	1.88 (s)	18.9	1.87 (s)
18	19.0 CH_3	1.41 (t, 3.9)	18.5	1.31 (s)
19	10.0 CH_3	1.75 (s)	10.1	1.78 (s)
20	63.8 CH_2	3.64 (dd, 7.6, 4.7)	64.9	3.93 (d, 7.2)
1'	119.3 C	-	117.3	-
2'	135.3 CH	5.81 (d, 15.9)	122.6	5.75 (d, 15.2)
3'	128.2 CH	7.39 (m)	135.1	6.73 (dd, 15.2, 11.2)
4'	127.2 CH	6.20 (m)	128.6	6.13 (m, 11.2, 14.8)

5'	129.9 CH	6.6 (m)	137.5	6.34 (dd, 14.8, 10.4)
6'	127.2 CH	6.16 (m)	130.3	6.09 (m, 10.4, 14.8)
7'	141.5 CH	5.97 (m)	137.1	5.78 m, 14.8, 7.6)
8'	36.1 CH ₂	2.1 (q, 8.7, 15.3)	31.5	2.09 (d)
9'	22.7 CH ₂	1.44 (m)	22.5	1.43 (m)
10'	13.8 CH ₃	0.92 (t, 7.4)	13.9	0.92 (t)
1''	165.9 C	-	165.6	-
2''	135.6 C	-	129.9	-
3''-7''	126.3 CH	7.72 (m)	129.7	7.90 (d, 7.2)
4''-6''	128.2 CH	7.39 (m)	128.8	7.46 (t, 7.6)
5''	129.9 CH	7.59 (m)	133.5	7.59 (s)

The analysis of the ¹H NMR, ¹³C NMR, and HSQC spectral data enabled the correlation of protons to their respective carbon bonds (Supplementary 3.34). The ¹H –¹H COSY spectrum showed an interaction between protons at δ 5.81 (H-2'), δ 7.39, (H-3'), δ 6.20 (H-4'), and δ 6.6 (H-5'), confirmed the location of these protons in the alkyl chain. The interaction between H-1 and CH₃-19 assisted with the assignment of proton at position of protons at δ 7.31, δ 3.93, and δ 1.75 were assigned to H-1, H-10, and H-19, respectively. The relative stereochemistry of this compound was based on comparison with literature review and the coupling constants. The coupling constant of 4.5 Hz observed for H-1 suggested that H-1/CH₃-19 are axial-equatorial. The large coupling constant observed at H-2' of 15.9 Hz confirmed the trans-geometry in the alkyl chain of this proton.

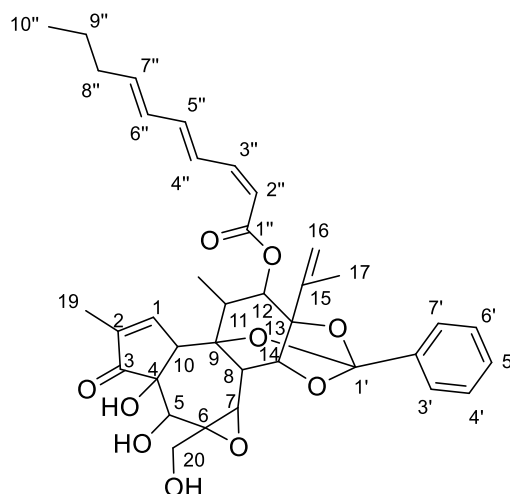


Figure 3.36: The structure of gniditrin

The proton NMR of the compound showed four methyl resonances at chemical shifts δ 1.88 (s, H-17), δ 1.75 (s, H-19), δ 1.41 (d, 7.2, H-18) and δ 0.92 (t, 7.4, H-10'). The spectrum also showed an aromatic system showed by signals at δ 7.72 (1H, m, H-3'-H-7'), δ 7.39 (1H, m, H-5') and δ 7.39 (2H, m, H-4'-H-6'), two aliphatic methylene protons at δ 2.1 (2H, q, 8.7, 15.8 H-8''), δ 1.44 (2H, m, H-9''). The characteristic presence of an isopropenyl moiety at δ 1.88 (H-17) and δ 5.02 (H-16) was also observed. Due to insufficient quantity of the purified compound, the ^{13}C NMR could not be obtained. However, the ^1H NMR spectra of gniditrin in comparison with published proton NMR was sufficient to give a tentative confirmation of what the compound could be as shown in Table 3.7.

Table 3. 8: Comparison of ^1H and ^{13}C chemical shifts of isolated gniditrin recorded using a 500 MHz NMR in with published data in CDCl_3

^1H NMR spectra of gniditrin in Hz in CDCl_3-d_1		
Position	Isolated gniditrin δ (ppm), J in Hz	Published data for gniditrin in CDCl_3-d_1 (^1H , 500 MHz) ^[26,27]
1	7.59 (1H, s)	7.48 (m)
2	-	-

3	-	-
4	-	-
5	4.27 (1H, s)	4.15 (s)
6	-	-
7	3.48 (1H, s)	3.53 (m)
8	3.80 (1H, dt (5.7, 12.5))	3.55 (m)
9	-	-
10	3.97 (1H, m)	3.84(m)
11	2.51(1H, m)	2.44 (m)
12	5.10 (1H, m)	5.03 (m)
13	-	-
14	4.92 (1H, s)	4.83 (d, 2.5)
15	-	-
16	5.02 (2H, d, 4.12)	4.94 (m)
17	1.88 (3H, s)	1.79 (s)
18	1.41 (3H t, 3.9)	1.29 (d, 7.3)
19	1.75 (s)	1.68 (m)
20	3.63 (2H, m)	3.84 (m)
		3.75 (d, 12.4)
1'	-	-
2'	-	-
3'	7.72 (1H, dd, 4.9, 1.96)	7.64 (m)
4'	7.39 (1H, d, 6.2)	7.30 (m)
5'	7.39 (1H, d, 6.22)	7.30 (m)
6'	7.39 (1H, d, 6.22)	7.30
7'	7.59 (1H, s)	7.64
2''	5.75 (1H, m)	5.71 (d, 15.2)
3''	7.39 (1H, d, 6.22)	7.16 (dd, 15.2, 11.2)
4''	6.17 (1H, m)	6.11 (dd, 14.9, 11.3)

5''	6.56 (1H, m)	6.46 (dd, 15.2)
6''	5.95 (1H, m)	6.03 (dd, 15.2, 10.0)
7''	5.10 (1H, m)	5.87 ((m)
8''	2.1 (2H, q, 8.7, 15.3)	2.03 (m)
9''	1.44 (m)	1.36 (m)
10''	0.92 (t, 7.4)	0.83 (t, 7.3)

3.8. The bioactivity of isolated daphnane diterpenoids

3.8.1. The *in vitro* anti-HIV activity of the isolated daphnane diterpenoids

The compounds yuanhuacine A, yuanhuajine, gniditrin and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin were evaluated for their anti-HIV activity *in vitro* at different concentrations of 5.00 to 0.08 µg/ml using a 2-fold serial dilution as shown in Figure 3.37. Yuanhuajine showed viral inhibition of 80.0±2.9% and 70.2±8.1% at 5 and 2.5 µg/ml, respectively. While gniditrin only showed inhibition activity of 66.7±10.2% at 5 µg/ml. Yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin inhibited the viral replication equally well at all test concentrations by 66.8±9.9% (yuanhuacine A) and 97.7±18.2% (mixture of yuanhuacine and cis 2''/3'' isomer of gnididin) at 0.08 µg/ml. Both yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin portrayed anti-HIV activity similarly with the positive control at low test concentrations.

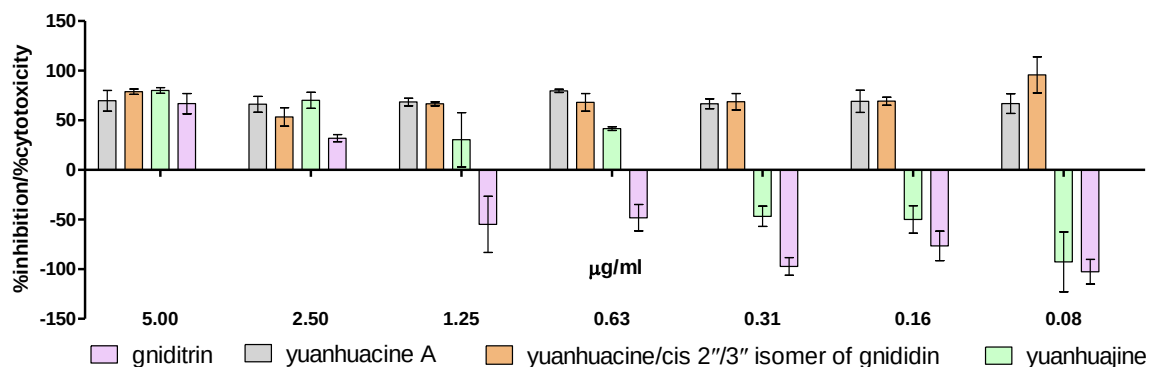


Figure 3.37: *In vitro* anti-HIV activity of the isolated compounds: yuanhuacine A, mixture of yuanhuacine and cis 2''/3'' isomer of gnididin, yuanhuajine and gniditrin at different test concentrations.

To rule out interference by toxicity, the cytotoxicity of the compounds was monitored at different test concentration as shown in Figure 3.38. The compounds displayed less than 50% cellular cytotoxicity at the test concentrations. Cytotoxicity is considered to be one of daphnane diterpenoids side effects. Having daphnane diterpenoids compounds with these properties yields to high selectivity index.

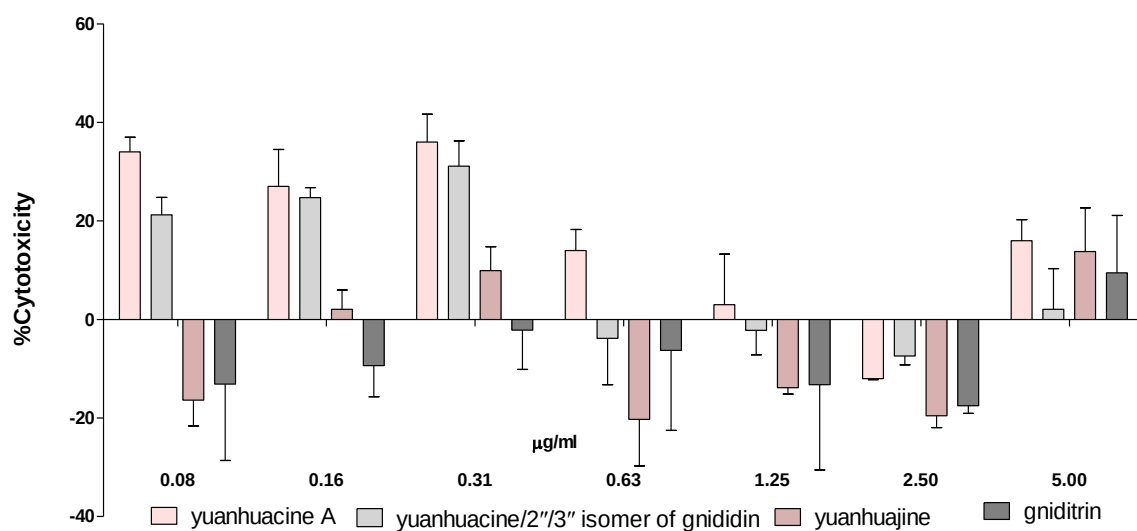


Figure 3.38: The cytotoxicity activity of yuanhuacine A, mixture of yuanhuacine and cis 2''/3'' isomer of gnididin, yuanhuajine and gniditrin.

Yuanhuacine A showed enhanced potency in comparison with yuanhuajine and gniditrin, while the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin showed enhanced potency

compared to yuanhuacine A. The enhanced anti-HIV activity in the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin could be attributed to the synergistic effect of the two compounds. Additionally, yuanhuacine is a known HIV inhibitor, with different therapeutic uses and adverse drug reaction, thus the compound was not tested further for its anti-HIV activity.^[22,26] However yuanhuacine A is a single compound entity and could provide greater selectivity in biological assays with improved therapeutic indices as a result the compound was further tested for its anti-HIV activity in an *ex-vivo* model.^[6,29–31]

3.8.2. The *ex vivo* anti-HIV activity of yuanhuacine A in peripheral mononuclear cells

The anti-HIV activity of yuanhuacine A in human peripheral mononuclear cells (PBMCs) was investigated using a subtype C viral strain from Ethiopia as shown in Figure 3.39. The *ex vivo* experiment was carried out by the Southern Research organisation (project number 16174.01). The nucleoside reverse transcriptase inhibitor AZT was used as a control with a measured IC₅₀ of 0.007 μ M and IC₉₀ of 0.05 μ M. The compound, yuanhuacine A demonstrated activity comparable to that of AZT, with an IC₅₀ and IC₉₀ of 0.03 and 0.09 μ M, respectively. It further showed excellent selectivity over cytotoxicity with a selectivity index of > 500. PBMC's are a source of a wide range of complex cells such as lymphocytes and monocytes as well as CD4⁺ cells, this variety of cells in the activity of yuanhuacine A could lead to other effective mechanisms that is essential in HIV treatment.

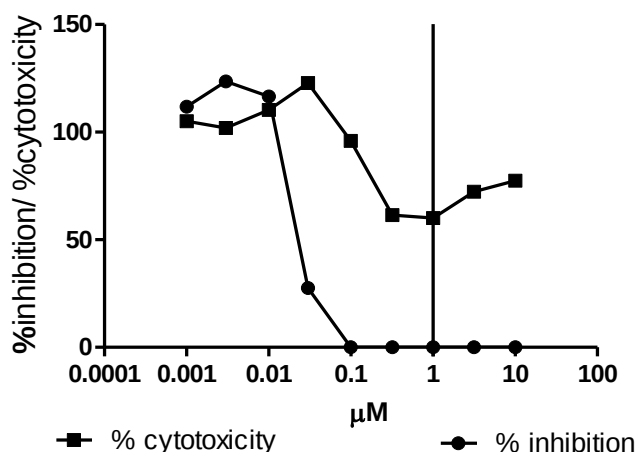


Figure 3. 39: Dose response and cytotoxicity curve of yuanhuacine A in PBMC's. The compound has an IC_{50} of 0.03 μ M and a selectivity index SI > 500.

3.8.3. The *in vitro* human ether-a-go-go channel inhibition by yuanhuacine A

The human ether-a-go-go gene (hERG, Kv11.1) K^+ channel plays an important role in cardiac electrophysiology and in drug safety.^[3] The inhibition of hERG is reported to be the most common mechanism for numerous non-anti-arrhythmic compounds that produce the Torsade's de Pointes (TdP) associated with QT prolongation.^[3,21] The hERG inhibition assay for this study was performed at Metrion Biosciences. This necessitates the investigation of *in vitro* drug-hERG interactions as it has become an important part of modern safety pharmacology.^[3,21]

The *in vitro* human ether-a-go-go (hERG) inhibition of yuanhuacine A tested at different concentrations is shown in Figure 3.40. The compound showed current % inhibition values of 3.19, 16.65, 18.41 and 32.29 at 0.02, 0.2, 2 and 20 μ M, respectively. While verapamil showed a current inhibition with an IC_{50} value of 0.61 μ M. Compounds that fail to block the channel by >40% at the high test concentration, their IC_{50} values are regarded to be greater than the top concentration, which is 32.29% in the case of yuanhuacine, resulting in an IC_{50} > 0.02 μ M for the compound. These results suggest that yuanhuacine A does not have hERG channelling properties (Supplementary data 3).

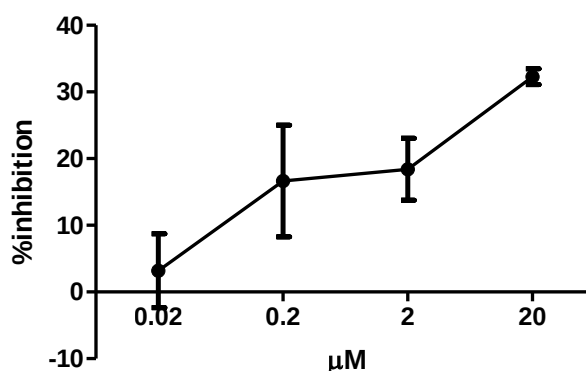


Figure 3. 40: hERG channel inhibition activity of yuanhuacine A with an IC_{50} >20 μ M.

3.8.4. Latency reversal activity of isolated compounds

The compound yuanhuacine A as well as the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin were investigated for their *in vitro* HIV latency reversal activity using J-Lat 10.6 cell line. The J-Lat 10.6 cells were derived from the Jurkat T cell line but contain a latently-infected, GFP-tagged HIV provirus.^[22] The treatment of cells with LRAs for 24 hours induced production of viral proteins and GFP, which was monitored by flow cytometry. The control PKC activator prostratin induced near-maximal GFP expression in $66.0 \pm 5.7\%$ of cells at $2.5 \mu\text{M}$ as shown in Figure 3.41A. In contrast, both yuanhuacine A and the isomeric mixture exhibited similar levels of latency reversal ($65.5 \pm 6.3\%$ and $58.2 \pm 4.7\%$, respectively) at $0.15 \mu\text{M}$, indicating ~16.7-fold enhanced activity over prostratin.

When the J-Lat 10.6 cells were treated in presence of $1 \mu\text{M}$ of the pan-PKC inhibitor Gö 6983, there was an observed reduction in latency reversal by $74.7 \pm 17.3\%$ due to $2.5 \mu\text{M}$ prostratin as shown in Figure 3.41B. These results are consistent with prostratin's function as a PKC activator. In contrast, the latency reversal induced by $0.1 \mu\text{M}$ Panobinostat, a control LRA that acts by histone deacetylase inhibition was unaffected by Gö 6983, consistent with previous observation. However, similar to prostratin, Gö 6983 inhibited $70.5 \pm 12.0\%$ and $71.5 \pm 11.9\%$ of latency reversal induced by $0.5 \mu\text{M}$ of yuanhuacine A or the isomeric mixture, respectively (Figure 3.41B), indicating that both function the compound and the isomeric mixture act as members of the PKC activator class of LRAs.^[32-34] Similar results were observed when cells treated with prostratin, yuanhuacine A, or the isomeric mixture, but not panobinostat, were additionally treated with $2 \mu\text{M}$ enzastaurin, a selective PKC β isoform inhibitor (respective $53.7 \pm 22.3\%$, $55.2 \pm 25.6\%$, and $56.0 \pm 27.0\%$ inhibition of latency reversal), indicating that, like other diterpenes, yuanhuacine A and the isomeric mixture function in large part through activation of these PKC isoforms.^[35]

The effects of yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin on cell viability was assessed on parental Jurkat cells lacking HIV provirus. The cells were

treated with the compounds for 24 hours and then measured for cell viability using resazurin dye. Both compounds affected cell viability similarly to prostratin as shown in Figure 3.41C. The treatment of Jurkat cells with 0.5 μM prostratin resulted in an average of $56.0 \pm 10.0\%$ viability (mean $\pm$ SD) relative to cells treated with 0.1% DMSO vehicle control, while treatment with 1.5 μM of yuanhuacine A or the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin resulted in $51.2 \pm 11.2\%$ and $58.5 \pm 15.9\%$ viability, respectively. These results indicate that the daphnane diterpenoids induce more latency reversal at similar concentrations to prostratin without additional loss of cell viability.

The compound's ability to activate T cells, Jurkat cells were next incubated with compounds for 24 hours and stained for the CD69 T cell activation marker. As expected, 2.5 μM prostratin induced an average $73.2 \pm 1.3\%$ (mean $\pm$ SD) CD69-positive cells (Figure 3.41D), consistent with prostratin's role as an activator of PKC and T cells. In contrast, cells treated with 0.1 μM Panobinostat, minimal CD69 expression was observed ($1.5 \pm 1.3\%$ positive cells). However, when the cells were treated with 0.5 μM of yuanhuacine A or the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin, widespread CD69-positive cells were observed ($75.4 \pm 2.2\%$ and $74.5 \pm 1.3\%$, respectively; Figure 3.41D), these results are consistent with yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin inducing T cell activation as PKC activators.

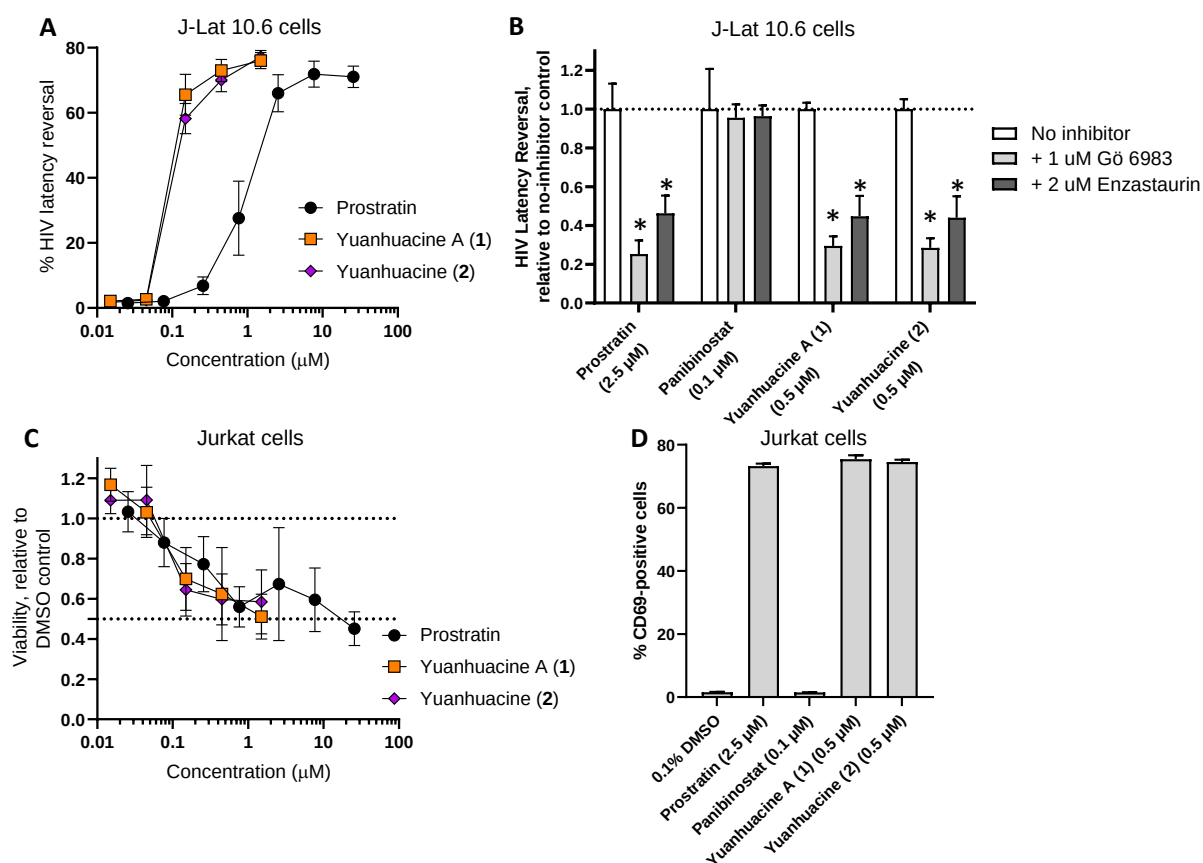


Figure 3. 41: A, the effects of yuanhuacine A and yuanhuacine (mixture of yuanhuacine and the cis 2''/3'' isomer of gnididin) on HIV provirus expression in J-Lat 10.6 T cells following 24 hours incubation, as measured by GFP reporter expression by flow cytometry. B, Effects of pan-PKC inhibitor Gö 6983 (1 μM) and PKC β inhibitor enzastaurin (2 μM) on LRA-induced latency reversal. C, Effects of compounds on cell viability in Jurkat T cells after 24 hours, as measured by resazurin dye. D, Effects of compounds on Jurkat T cell activation, as measured by CD69 expression, following 24 hours incubation with compounds. Results denote the mean ± SD of at least 3 independent experiments. *, p < 0.01 by Student's two-sided t test.

The HIV latency reversal activity of yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin (labelled as yuanhuacine) was also investigated in primary cells obtained directly from 3 HIV-infected, cART-suppressed patients. This was done to establish HIV latency reversal in a primary cell context as shown in Figure 3.34A. Briefly, 2×10^6 CD4+ T-cells per donor were cultured in the presence of 0.1% DMSO, anti-CD3/CD28 dynabeads (a positive control CD4+ T cell activator), 10 μM prostratin, or 1.5 μM of yuanhuacine A or the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin. After 24 hours of incubation, the cell viability was assessed by trypan blue staining, and absolute viral RNA in each culture supernatant was determined by qRT-PCR.^[36] A donor-to donor variability was observed, which

is consistent with previous data. Despite this, both yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin were generally well tolerated by CD4+ T cells as measured by direct cell counting with trypan blue (average 96.4 ± 32.2 and $79.6 \pm 41.5\%$ viability respectively, compared to $65.9 \pm 12.4\%$ viability in the presence of prostratin as shown in Figure 3.42A).

When the compounds were assessed for total copies of supernatant viral RNA, a baseline average of 32.6 ± 43.3 viral copies/mL across 3 donors was observed, with 1 donor lacking viral RNA above the limit of detection of this assay (10 copies/mL; Figure 3.42B, dotted line). In contrast, anti-CD3/CD28 increased viral RNA expression from all three donors by an average 8.8-fold over unstimulated cells with an average of 287 ± 367 copies/mL. For cells treated with 10 μ M prostratin, an increased viral RNA across all donor samples by an average 23.2-fold increase of 755.5 ± 1144.2 copies/mL was observed. Similarly, 1.5 μ M of yuanhuacine A or the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin induced comparable increases in viral RNA to anti-CD3/CD28 which approached the activity of prostratin despite a much lower concentration. An average 10.8-fold increase in viral RNA for yuanhuacine A (average 352.5 ± 430.4 copies/mL) and 9.1-fold increase for the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin (average 297 ± 267 copies/mL as shown in Figure 3.42B). Taken together, these results indicate that both yuanhuacine A the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin are potent activators of HIV latency reversal *ex vivo* in addition to potent inhibitors of viral replication.

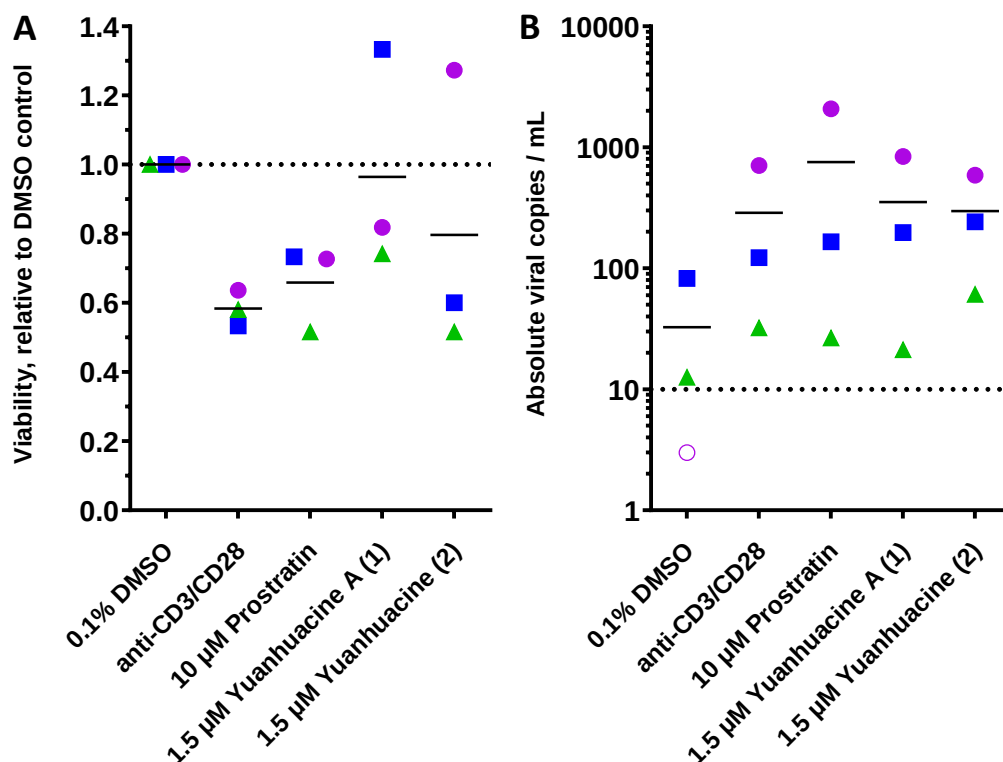


Figure 3. 42: Effects of 10 μ M prostratin and 1.5 μ M of yuanhuacine A or the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin (labelled as yuanhuacine) on cell viability (A) and absolute viral RNA copies/mL of supernatant (B) in primary CD4+ cultures from 3 cART-suppressed donors with HIV following 24 hours treatment. For both panels, shapes and colors denote individual donors. In panel B, dotted line denotes limit of detection, and empty shape denotes no viral RNA detected.

The correlation between the chemical structure and the anti-HIV activity of all isolated daphnane diterpenoids is that all compounds have a poly-hydroxyl group at positions C-3, C-4, C-5, C-20, with an ortho-ester motif at positions C-9/C-13/C-14 and 6 α epoxy ring system.^[32] The compounds all have an α - β unsaturated ketone group at C-3.^[26] These similar functionalities may be attributed to the anti-HIV activity seen in the compounds especially at higher test concentrations. The compounds also have an alkyl chain and a phenyl group attached at positions C-1' and/or C-12.^[16]

However, there is a difference in the attachment of the benzyl ring and the alkyl chain in the compounds. Yuanhuacine A, yuanhuajine and yuanhuacine all have the same substitution patterns in the same region.^[23] The difference between the three compounds is in the aliphatic

chain, whereby yuanhuacine has an extra CH₂-CH₂, while, yuanhuajine has an extra CH-CH in its alkyl chain compared to yuanhuacine A.^[27] The extra double bond in yuanhuajine could contribute to the compound's weak anti-HIV activity in lower test concentrations. The anti-HIV activity of the pure yuanhuacine has been widely reported for its anti-HIV activity, this also confirms the anti-HIV activity observed in the mixture of the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin. Other compounds isolated have the phenyl system attached to the orthoester and these include gniditrin and the cis 2''/3'' isomer of gnididin which was isolated for the first time in this study. In this study the anti-HIV activity of gniditrin was very weak and this could be due to the attachment of these functional groups in comparison to other compounds.

The inactivity possessed by lasiocephalin and lasioerin as non-daphnane diterpenoids confirms the anti-HIV activity in the plant extract is due to daphnane diterpenoids.^[30] Yuanhuacine A was chosen for further anti-HIV studies as it showed good inhibition activities and was obtained as a single entity.

3.9. Conclusion

The study reported for the first time diterpenoids present in *G. sericocephala* roots based on the traditional mixture used in the treatment of HIV. The purification of these compounds was made successful by following multiple purification steps. These include the HPLC microfractionation which guided the purification process, as it localised the activity leading to microfractions 25, 26, 27, 28 and 29 being the only fraction that inhibited *in vitro* virus replication by $52.0 \pm 0.9\%$, $71.0 \pm 5.9\%$, $83.9 \pm 9.6\%$, $103 \pm 2.1\%$, $101 \pm 2.0\%$ and $91.6 \pm 2.8\%$, respectively, from a total of 30 microfractions. The chemical analysis of the active microfractions using UPLC-QTOF-MS/MS revealed the presence of daphnane diterpenoids which were further purified by repetitive. The anti-HIV activity in the DCM extract was successfully localised by HPLC directed fractionation. The UPLC-QTOF-MS/MS profile of the

combined microfractions showed the presence of coumarins and daphnane diterpenoids which are known for their anti-HIV activity. The daphnane diterpenoids corresponding to m/z 621.2669 (peak 1), m/z 647.2833 (peak 2 and 3), and m/z 649.3020 (peak 4) were tentatively identified yuanhuacine A, yuanhuajine, gniditrin and yuanhuacine and or cis 2''/3'' isomer of gnididin. This was based on the class of these compounds (daphnane diterpenoids) being known for having potent anti-HIV properties.^[27,35] These were hypothesised to be responsible for the observed anti-HIV activity in the microfractions and were prioritised for isolation and purification. The UPLC-QTOF-MS/MS of the microfractions also revealed that the compounds were medium to non-polar based on their retention time. The inactive major peaks in the extract were successfully isolated as biomarkers and later identified using 1D and 2D NMR as lasioerin and lasiocephalin.

The preparative purification of the active fractions resulted in the isolation of five daphnane diterpenoids (yuanhuacine A, yuanhuacine, cis 2''/3'' isomer of gnididin, yuanhuajine and gniditrin) with viral inhibition properties as well as HIV latency reversal activity. The compound yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin showed HIV replication inhibition (*in vitro*) by > 80% at 0.08 µg/ml. Furthermore, yuanhuacine A inhibits a subtype C virus replication in PBMC with an EC₅₀ of 0.03 µM without cytotoxicity. The compound, yuanhuacine and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin also reverse HIV latency *in vitro* 16.7-fold lower concentrations than control prostratin and also reverse latency in primary CD4⁺ T cells from cART-suppressed donors with HIV similar to prostratin but at 6.7-fold lower concentrations. These results suggests that *G. sericocephala*, yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin act as anti-HIV agents to improve cART efficacy and support HIV cure efforts in resource-limited regions.

3.10. References

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Chapter four

Extraction optimization and quantitative analysis for the development of Product Nkabinde

4.1 Introduction

The special chemistry offered by natural products over their synthetic counterparts provides human-kind with both advantages and challenges for drug discovery processes, arising from the enormous diversity in their scaffolds and structural complexity.^[1] In African traditional medicine, medicinal plants are often used in combinations to strengthen the effectiveness of the herbal medicine. However, the active ingredients need to be identified and characterised.^[2] Concurrently, the identification of the single pure compounds from medicinal plants for the treatment of different ailments can be a daunting process, which sometimes leads to the loss of activity due to degradation of compounds during the purification steps.^[3] The synergistic effect compounds have as a mixture plays a crucial role in most herbal extracts as the mixture of these compounds may increase absorption of the medicine in the body or have a different mode of action all together such as an immune booster.^[4-6]

The challenges that arise when using traditional medicine based compounds can be overcome by having the active compounds in a mixture with other compounds such as an extract, or fraction to assist in drug delivery while retaining the activity.^[6] This approach has been practised and deemed reliable as long as the active compound is known and available in sufficient concentration in the mixture.^[7,8] The chemical composition and quantity of detected compounds depends to a large extent on the sample preparation methods chosen and use of a fast, reproducible and selective extraction method in acquiring compounds of interest. ^[9-11] Major efforts are put into optimizing sample extraction and separation as well as instrumental conditions to measure certain compounds in plant extracts by complying with regulatory processes in place, assessment of safety and efficacy, as well as quality control.^[7,12,13]

There are several stages that need to be taken into consideration before extraction of phytochemicals from medicinal plants and these include plant authentication before any work is done on them for quality control purposes. The use of the correct plant part plays an important role in the development of herbal extract as plants tend to contain different classes of compounds in different parts.^[14] The time, season and place of collections also affect the chemistry of the plants which in turn could affect the quality. Drying techniques and conditions are also of paramount importance as they could disintegrate the compounds if the plant is exposed to excess heat.^[15-16] Solvent selection is one of the important stages in plant extraction due to the solubility compatibility between compounds of interest and the solvent of choice as polar compounds would require to be extracted with polar solvents such as methanol and sometimes water, non-polar compounds would require non-polar solvents such as hexane and dichloromethane.^[17] However, solvents such as methanol and hexane are not ideal solvents for pharmaceutical production of herbal medicines as they have been deemed unsafe for human consumption.^[16] The use of solvents such as ethanol and water are deemed safe for human consumption and would be the ideal solvents in herbal extract production.^[17-18] Extraction time is another important parameter as the compounds of interest could not be extracted if the extraction time is shorter, on the other hands if there is over extraction time, non-essential compounds could also be extracted, and these could affect the efficacy of the extract.^[18] Hence, trial and error extraction needs to be performed to achieve the desired results before the scale up extraction.^[19-20]

4.2 Regulatory processes in herbal medicine production

An appropriate regulatory system in the development of herbal traditional medicines needs to be followed as it ensures and promotes the quality, safety and efficacy of the ingredients of a standardised herbal medicine.^[21] In many developing countries including South Africa a high number of the population, more than 80%, still rely on traditional medicine as their primary source of healthcare. These medicines are reported to treat a wide range of ailments ranging

from cancer, hypertension, malaria, HIV and many more. However, their documented use is insufficient as it is largely based on the oral tradition of passing down information from family to family which increases the risk of losing the information being passed down from generation to generation throughout the years.^[24-25]

In order to fulfil the existing high demand of herbal extracts, established herbal processing platforms are essential in the production phase of the herbal medicine.^[26-27] These platforms have the capacity to produce high yields of phytochemical production with consistent quality which is dependent on a marker compound. However, there are some difficulties faced with having consistent quality and quantity of the herbal extract production, hence, trial an error methods are usually performed to alter some parameters in order to achieve the desirable standardised herbal extract that is enriched with compounds of interest which will also meet the standard of the regulatory processes.^[28-30] The good practices of manufacturing processes offered by these established platforms play a big role in the quality control and standardization of the herbal extracts.^[31-33]

4.3 Upscale and pilot scale production of herbal extracts

One of the requirements for a developed product to reach market is that its efficacy and safety needs to be known.^[25] Even though these have been provided at laboratory scale, it is a necessity to confirm if whether the efficacy was retained during the industrial scale process as many factors could have played a role in losing the known efficacy. Hence, the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four plant mixture produced from the semi-preparative scale-up production were biologically screened for their anti-HIV activity and latency reversal activity to ensure retained activity.^[25-27]

The extraction of phytochemicals from lab scale to industrial scale has been achieved by following different extraction methods to optimize the chemical profile, quantity of extract, as well as retention of the bioactivity of the extract. However, the deviation from the lab extraction process has been shown to result in loss of activity and different chemical profile. To avoid

this, the industrial scale process in this study tried by all means to mimic the industrial scale process.

The laboratory scale extraction method of the 50% ethanol extract of the four-plant mixture and the ethyl acetate fraction of *G. sericocephala* were used to determine the industrial scale extraction projections. The projections were used to determine the theoretical yields to be obtained if a total of 80 kg plant material made up by 20 kg of each plant material was extracted to obtain the 50% ethanol extract of the four-plant mixture and 20 kg was extracted for the development of the ethyl acetate fraction of *G. sericocephala* at industrial scale. The laboratory projections were made available to Afriplex Pty Ltd, a GMP approved herbal manufacturing company to upscale to production scale. Afriplex worked on the projections to simulate the expected extraction yield and losses based on the actual quantities of raw material provided to them.

4.4 Methods

4.4.1. Plant collection

A second batch of *S. birrea* stems, *G. sericocephala* roots, *S. italica* roots and *P. prunelloides* roots was collected and delivered by THP Nkabinde to be used in the semi-pilot scale extraction for the development of the products. The plants were collected from Tugela Ferry in Msinga region of KwaZulu Natal . THP Nkabinde delivered to the University of Pretoria dried and ground plant material. The amounts delivered are shown in Table 4.1.

Table 4.1: Quantities of delivered dried and ground plant material by TDr. Nkabinde

Plant name	Quantity delivered in kilogram (kg)
<i>S. italica</i> roots	20
<i>S. birrea</i> stem	20
<i>P. prunelloides</i> roots	20

<i>G. sericocephala</i> roots	40
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4.4.2. Semi-pilot scale extraction of *G. sericocephala* and the four plant mixture

The production scale extraction and development of the ethyl acetate fraction of *G. sericocephala* roots was carried out based on the laboratory scale extraction described in Chapter 2 (2.7.5). The production scale of *G. sericocephala* and the four plant mixture was carried out by Afriplex Pty Ltd ([https://Afriplex Pty Ltd .co.za/who-we-are/our-facility-and-quality-systems/](https://Afriplex_Pty_Ltd_.co.za/who-we-are/our-facility-and-quality-systems/)). The company is a manufacturing company that adheres to the pharmaceutical cGMP standards, the company was chosen due to its facilities having the capacity to handle large volumes of solvents.

4.4.2.1 Ethanol extraction of *G. sericocephala* and liquid-liquid partitioning between water and ethyl acetate

A total of 4.647 kg of *G.sericocephala* roots was extracted by maceration by Afriplex Pty Ltd Pty Ltd. The plant material was added to a 100 L container containing 48 kg ethanol (96.4% v/v) and stirred using a stainless-steel paddle stirrer for 10 minutes. The mixture was extracted for 10 days and mixed for 10 minutes every day to increase extraction yields. After 10 days the solvent was removed from the plant material by pressing with a small hydraulic press to maximizing extraction yield. Impurities from the extract were removed through filtration of the extract through a microfiltration system. The filtered extract was stirred for 3 minutes and evaporated at 50°C in a rotary-evaporator to produce a concentrated ethanol extract as shown in Figure 4. 1.

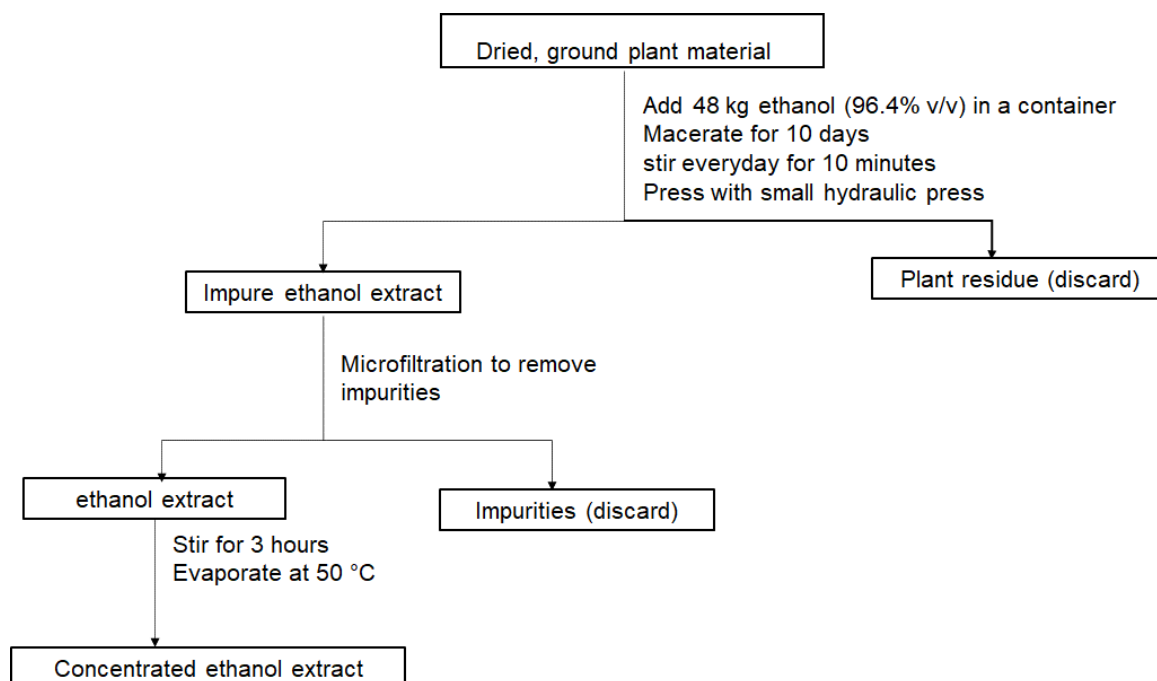


Figure 4. 1: The ethanol pilot scale extraction of *G. sericocephala* roots by Afriplex Pty Ltd

The concentrated ethanol extract (395.73 g) and an additional 5 kg of reverse osmosis (RO) water used to remove the slurry from the rotary evaporator, were extracted with 63 kg of ethyl acetate and stirred for 10 minutes with a stainless-steel paddle stirrer. The extract was left to stand for 3 days while being mixed for 5 minutes each day to increase extraction efficiency. The ethyl acetate soluble layer was removed after 3 days of maceration and placed in a pre-weighed 200 L container. The same amount of ethyl acetate 63 kg was added to the plant material after the removal of the ethyl acetate and macerated for 3 days with 5 minutes of mixing each day and removing the ethyl acetate extract. The same process was repeated for the final time and the ethyl acetate was pumped into a 200 L container with the previously used ethyl acetate extracts. The combined fractions were later placed in a separating funnel to remove the water slurry from the ethyl acetate solvent and evaporated in a rotary evaporator as shown in Figure 4.2.

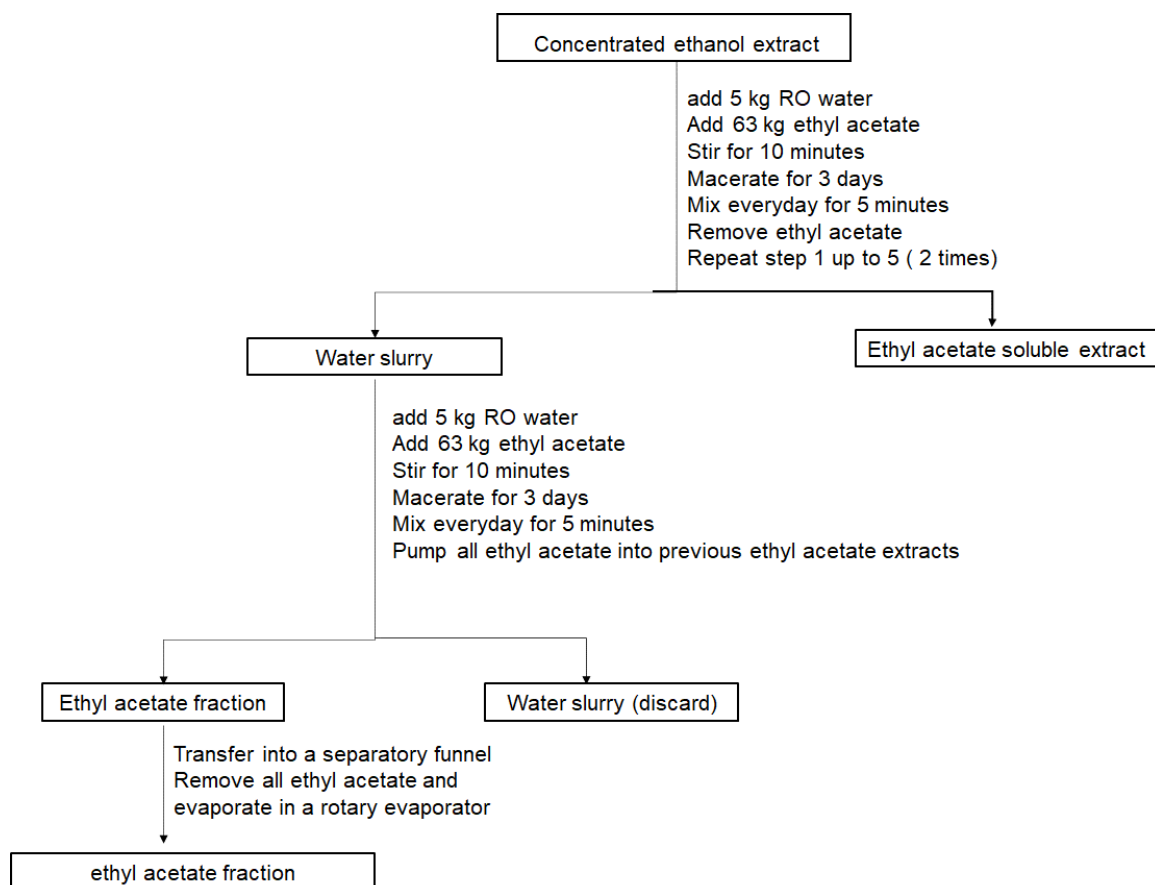


Figure 4. 2: Liquid-liquid extraction of the ethanol extract of *G. sericocephala* between ethyl acetate and water.

4.4.2.2 Extraction of the four plant mixture using 50% ethanol

The four plant mixture (66.8 kg) consisting of 16.7 kg of each plant (*S. italica*, *S. birrea*, *P. prunelloides* and *G. sericocephala*) was extracted using 325,6 kg ethanol and 142 kg of RO water (making a total of 467.6 kg of 50% ethanol) . The plant material was extracted by stirring in a mobile tank using an air pump for three hours. The extracted plant material was left to stand for an additional three hours, after which the solvent was pumped off into a clean intermediate bulk container (IBC) container. The extraction process was repeated twice more and after the third extraction, the solid was separated from the liquid using a small hydraulic press and the liquid filtered using by microfiltration. The extract was concentrated by at 50 °C

in a vacuum, and later evaporated for the second time in a rotary evaporator as shown in Figure 4.3.

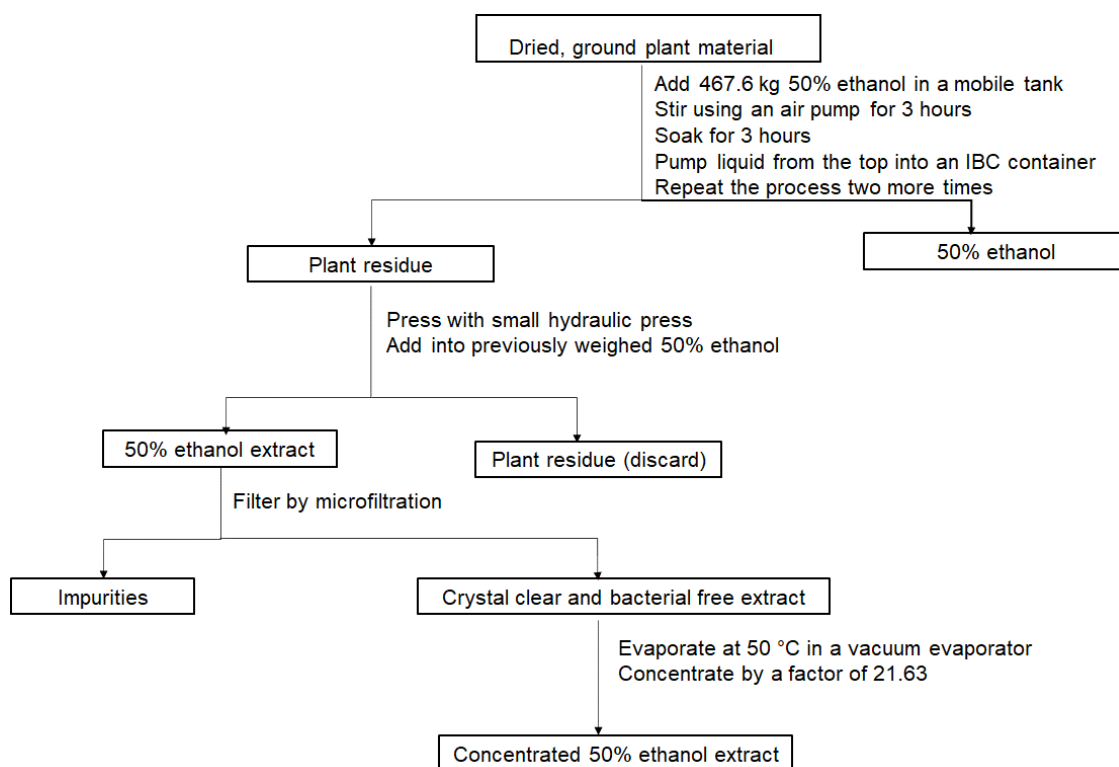


Figure 4. 3: Pilot scale extraction of the four plant mixture with 50% ethanol by Afriplex Pty Ltd

Attempts at further concentration of the thick slurry by spray drying was unsuccessful and was delivered to the University of Pretoria. A small quantity (20 mg) of the 50% ethanol extract of the four plant mixture was further dried using a Genevac for further testing of its anti-HIV activity and latency reversal activity.

4.4.2.3. Further drying of the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four plant mixture

The ethyl acetate fraction of *G. sericocephala* roots delivered by Afriplex Pty Ltd was further dried in the laboratory using a rotary evaporator to a fine powder and stored in a pre-weighed container. The concentrated 50% ethanol extract of the four plant mixture was received in a 25 L container consisting of two layers that was further dried in an oven at 80 °C. Prior to

drying, the concentrated extract separated in two layers. The two layers (top and bottom layer) were separately analysed by UPLC-MS. The dried ethyl acetate fraction from *G. sericocephala* and the oven dried 50% ethanol extract were later bioassayed for their anti-HIV activity and HIV latency reversal activity.

4.4.2.4. Quality control analysis of the ethyl acetate fraction from *G. sericocephala* and the 50% ethanol extract of the four plant mixture.

The quality analysis including physical, chemical, heavy metal, pesticides, aflatoxin and microbial specifications was performed on the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four-plant mixture. The European Pharmacopoeia, United States Pharmacopoeia, WHO and Afriplex Pty Ltd inhouse methods (SOP-QC023) were followed. ^[25] The parameters, specifications and test methods performed for QC analysis by Afriplex PTY LTD on the samples is shown in Table 4.2.

Table 4. 2: Parameters, specifications and test methods performed for QC analysis on the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four plant mixture

Parameter	Specification	Test methods
Physical		
Characteristics	Dark tincture	WHO/Pharm/92.599/rev 1 page 10
Chemical		
Identification by FTIR	≥95% similarity to reference	SOP-QC023
Identification by TLC	The R _f values of the sample correspond to the R _f values of the reference	Ph.Eur.2.2.27,SOP-QC142
pH	1.5-3.0	Ph.Eur.Appendix VL
Total solids	TBC	Ph.Eur.7.2 Method I
Specific gravity @ 20 °C	0.80 – 1.00 g/mL	USP 841 Method I
Residual solvents	Ethanol	GC-FID
Heavy metal		
cadmium	<1.0 ppm	Ph.Eur.2.4.8
Lead	<5.0 ppm	Ph.Eur.2.4.8
Mercury	<5.0 ppm	Ph.Eur.2.4.8
Arsenic	<1.0 ppm	Ph.Eur.2.4.8
Pesticides		
pesticides	Meets Ph.Eur.2.8.13 requirements	Ph.Eur.2.8.13
Aflatoxins		
Aflatoxins B1	<2 µg/kg	Ph.Eur.2.8.18
Aflatoxin B2	<2 µg/kg	Ph.Eur.2.8.18
Aflatoxin G1	<2 µg/kg	Ph.Eur.2.8.18
Aflatoxin G2	<2 µg/kg	Ph.Eur.2.8.18

Total aflatoxin (B1,B2, G1, G2)	<4 µg/kg	Ph.Eur 2.8.18
Microbial		
Total aerobic plate count	< 2000 cfu/g	Ph.Eur. Method 2.6.31; Ph.Eur. Method 2.6.12-13
Combined yeast and mould	< 100 cfu/g	Ph.Eur. Method 2.6.12-13
Bile tolerant gram negative bacteria	Absent/g	Ph.Eur. Method 2.6.12-13
<i>Staphylococcus aureus</i>	Absent/g	Ph. Eur. Method 2.6.12-13
<i>Escherichia coli</i>	Absent/g	Ph. Eur. Method 2.6.31; Ph. Eur. Method 2.6.12-13
<i>salmonella</i>	Absent/25 g	Ph. Eur. Method 2.6.31; Ph. Eur. Method 2.6.12-13

Ph.Eur. denotes European pharmacopoeia, USP denotes United States pharmacopoeia, WHO denotes world health organization. GC-FID denotes gas-chromatography-flame ionization detector

4.5 Biological assays

4.5.1. *In vitro* anti-HIV activity of the *G. sericocephala* ethyl acetate fraction and the 50% ethanol extract of the four plant mixture

The HIV inhibition activity of the ethyl acetate fraction of *G.sericocephala* and 50% ethanol extract of the four plant mixture were tested against pNL-NF plasmid using the DeCiPhR assay. The samples were tested at seven different concentrations of 5 µg/ml to 0.008 µg/ml following a 2-fold serial dilution. The known inhibitor efavirenz was used as a positive control. The detailed procedure of the assay described in section 2.8, Chapter 2.

4.5.2. *In vitro* latency reversal activity of the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four plant mixture

The Jurkat-derived, J-Lat 9.2 cells containing an *env* proviral genome were used to evaluate the latency reversal, viability and cellular activation of the ethyl acetate fraction of *G. sericocephala* roots and the 50% ethanol extract of the four-plant mixture in a dose response manner. Latency reversal activity was evaluated in parallel with a PKC activator prostratin and an HDAC inhibitor and was measure by GFP expression. The detailed latency reversal activity procedure is described in Supplementary data 4.3.

4.5.3. UPLC-QTOF-MS analysis of *G. sericocephala* ethyl acetate fraction and 50% ethanol extracts of the four plant mixture

The chemistry in plant extracts can differ from batch to batch, which could lead to the extract losing its therapeutic value. The chemical profile of the ethyl acetate fraction from *G. sericocephala* roots was investigated to identify the active compounds in the fraction and also to produce the chemical fingerprint of the fraction for future development of the fraction. The chemical analysis of the 50% ethanol extract of the four plant mixture was performed to investigate the presence or absence of the active compounds in the four plant mixture as the anti-HIV activity of the extract was associated with the presence of compounds from *G. sericocephala*.

The chemical analysis of the fraction and extract was performed using a Waters Acquity UPLC system (Waters Corp., MA USA) equipped with a binary solvent delivery system. The auto sampler was operated using MassLynx 4.1 software (Waters Inc., Milford, Massachusetts, USA) to obtain the chemical profile of the crude extracts. Instrument calibration was performed using sodium formate solution over a mass range of 50-1200 Da to an absolute mass accuracy of <0.5 mDa.

The separation of compounds was performed on a Waters BEH C18 reverse phase column (2.1 mm × 100 mm, 1.7 µm). The mobile phase consisted of water in 0.1 % formic acid (solvent A) and acetonitrile in 0.1 % formic acid (solvent B). The gradient elution was carried as follows: 3% solvent B (0-0.1 min) followed by an increase to 100% B between (0.3-14) an isocratic hold at 100% solvent B (14-16 min) and later optimized to 3% B (16.50-20.00 min). The flow rate was maintained at a 0.4 ml/min with an injection volume of 5 µl with a constant column temperature of 40 °C. The following MS source parameters were set for both positive and negative electrospray mode: source temperature of 100 °C, sampling cone voltage of 15 V, extraction cone voltage of 4.0 V, desolvation temperature of 400 °C, cone gas flow of 10.0 L/h, desolvation gas flow of 700 L/h and a capillary voltage of 2.0 kV.

All the samples were prepared to a final concentration of 1 mg/ml in 80% acetonitrile and were run in both positive and negative ionisation mode. Elemental composition, MS/MS fragmentation patterns, iFit values, mass error, Metlin, Mass bank, ChEBI (chemical entities of biological interest) and Metfusion were used for the tentative identification of compounds

Additionally, the UPLC-QTOF-MS chromatograms of lasioerin, lasiocephalin, yuanhuacine A, yuanhuacine (as a mixture of isomers), gniditrin and yuanhuajine obtained as described in Chapter 3 along with their MS/MS fragmentation patterns were used to confirm the presence of these compounds in the ethyl acetate fraction of *G. sericocephala*. These were prepared by dissolving 1 mg of each compound separately in 1 ml of acetonitrile and analysed using the same conditions described in section 4.5.4.

4.5.4. Quantification of yuanhuacine A in the herbal mixtures

In order to develop a single method that could detect, separate, and quantify yuanhuacine A in the ethyl acetate fraction and the ethanol extract of the four-plant mixture, a single run of 1 mg/ml of yuanhuacine A was first carried out using method described in section 4.5.3 and equipment?

4.5.5. Preparation of stock solution of yuanhuacine A

Yuanhuacine A isolated from the DCM extract of *G. sericocephala* with a purity of 97% (Chapter 3, section 3.6.13.3) was used as a working standard in this study. A working standard stock solution of yuanhuacine A of 100 mg/L was prepared by dissolving 0.5 mg of the compound in acetonitrile up to a volume of 5 ml in a volumetric flask. The standard solutions of different concentrations (20, 10, 5, 2.5 and 1 mg/L) were prepared by serial dilutions with an appropriate solvent of acetonitrile to achieve the concentration range needed for the development of the calibration curve for yuanhuacine A detection. The method was evaluated by determining the limit of detection and limit of quantification. The LoD and LoQ for each

analyte were calculated using the formulas $LoD = 3s / m$ and $LoQ = 10s / m$, where s stands for standard error of the mean and m corresponds to the slope.

4.6 Results and discussion

4.6.1. Semi-pilot scale extraction

The semi pilot scale extraction optimization of *G. sericocephala* was done to obtain a concentrated ethyl acetate fraction with retained bioactivity when compared to the laboratory scale fraction. The semi pilot scale extraction and liquid-liquid partitioning of the ethanol extract of *G. sericocephala* resulted in a dark brown concentrated liquid of the ethyl acetate fraction with an extraction yield of 1.527 kg at 6.72% total solids as a thick dark brown slurry. After further drying in the laboratory of the ethyl acetate fraction, the sample was reweighed to give 80 g with an extraction yield of 1.72%.

The semi pilot extraction optimization of the 50% ethanol extract of the four-plant mixture resulted in a dark brown liquid of approximately 50 L corresponding to 22.1 kg thick concentrate with an extraction yield of 77%. The extract was further concentrated to obtain approximately 25 L of the 50% ethanol extract and was later delivered to the University of Pretoria. The more concentrated form (25 L) separated into two layers, a bottom oily layer and a top tincture type layer. After combining and drying the two layers in an oven, a 2.8 kg dried ethanol extract of the four-plant mixture representing an extraction yield of 4.19%. was obtained. The drying of the four-plant mixture posed some challenges and would require in the future to be dried using an industrial freeze drier to completely remove all the liquid matter without exposing the extract to high temperatures.

4.6.2. Quality control parameters of the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four plant mixture

The dark brown concentrated tincture of the ethyl acetate fraction of *G. sericocephala* had a pH of 1.91 and total solids of 6.72%. A very dark brown and thick concentrated 50% ethanol extract of the four-plant mixture had a pH of 4.98 with total solids at 44.07%. The ethyl acetate

fraction of *G. sericocephala* was more acidic than the 50% ethanol extract of the four-plant mixture. The results from the quality control analysis of the two samples was performed by Afriplex PTY LTD is shown in Table 4.3. The heavy metal and aflatoxins levels in both samples were within specification as shown in Table 4.3. The ethyl acetate fraction and the 50% ethanol extract were also free from pesticides and microbial growth as shown in Table 4.3. In conclusion the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four-plant mixture passed all quality control parameters that are generally applicable to liquid concentrates.

Table 4.3: Quality control analysis performed for on the ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four plant mixture

Parameter	Quality control analysis of the ethyl acetate fraction	Quality control analysis of the 50% ethanol extract of the four-plant mixture
Physical		
Characteristics	the sample met acceptance criteria	the sample met acceptance criteria
Chemical		
Identification by FTIR	the sample was the first reference to be analysed by FTIR	the sample is the first reference to be analysed by FTIR
Identification by TLC	the sample met acceptance criteria	the sample met acceptance criteria
pH	1.91,	4.98
Total solids	6.72 % m/m,	44.07 % m/m
Specific gravity @ 20 °C	0.913 g/mL	1.09 g/mL
Residual solvents	N/A	4.742% v/v
Heavy metal		
cadmium	<0.01 ppm,	0.02 ppm
Lead	<0.01 ppm	<0.01 ppm
Mercury	<0.01 ppm	< 0.01 ppm
Arsenic	<0.01 ppm	0.01 ppm
Pesticides		
pesticides	non detected	none detected
Aflatoxins		
Aflatoxins B1	<1 µg/kg	<1 µg/kg
Aflatoxin B2	<1 µg/kg	<1 µg/kg
Aflatoxin G1	<1µg/kg	<1µg/kg
Aflatoxin G2	<1µg/kg	<1µg/kg
Total aflatoxin (B1,B2, G1, G2)	<4 µg/kg	<4 µg/kg
Microbial		
Total aerobic plate count	no growth/g	no growth/g
Combined yeast and mould	< no growth/g	< no growth/g

Bile tolerant gram negative bacteria	<10 cfu/g	<10 cfu/g
<i>Staphylococcus aureus</i>	no growth/g	no growth/g
<i>Escherichia coli</i>	no growth/g	no growth/g
<i>salmonella</i>	Absent/25 g	Absent/25 g

4.6.3. *In vitro* anti-HIV activity of *G. sericocephala* ethyl acetate fraction and the 50% ethanol extract of the four plant mixture

The *in vitro* anti-HIV activity of the ethyl acetate fraction of *G. sericocephala* roots is shown in Figure 4.4. The fraction showed anti-HIV activity in a dose response manner with an IC₅₀ value of 0.058 µg/ml. This indicated that the scaled-up process resulted in retention of its anti-HIV activity as the laboratory scale produced ethyl acetate fraction showed anti-viral activities of greater than 50% when tested at the lowest test concentration of 0.25 µg/ml, Not only did the fraction retain its activity, but the cytotoxicity levels were also very low (< 20%) at all test concentrations.

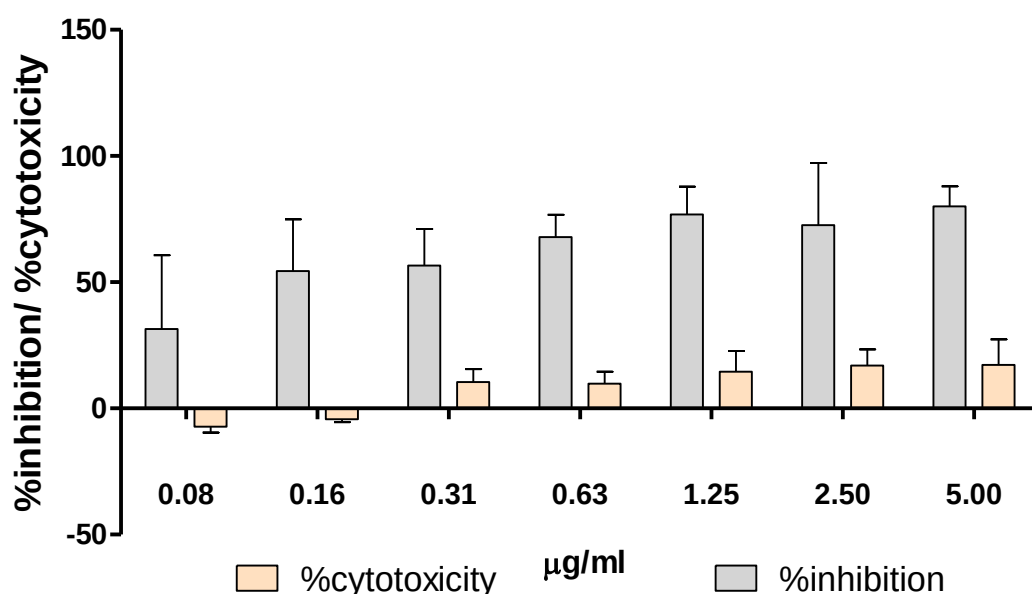


Figure 4.4: The *in vitro* anti-HIV activity of the ethyl acetate fraction of *G. sericocephala* roots

The *in vitro* anti-HIV activity of the production scale 50% ethanol extract of the four-plant mixture is shown in Figure 4.5. The extract only showed an anti-HIV activity of 60.03±7.3% at

5.00 µg/ml. In comparison to the laboratory scale extract, the anti-HIV activity in the production scale extract was lost as it was greater than 50% when screened at 1 µg/ml (Chapter 2, Figure 2.22). This could be attributed to the extraction of more polar compounds such as carbohydrates and peptides in large quantities during the pilot scale extraction, resulting in the dilution of the active compounds in the mixture. Another contributing factor could be exposure of the product to high temperatures during oven drying which is an indication that daphnane diterpenoids are not stable under high temperatures. Moreover, a different drying method would be recommended for further studies on this extract in order to retain the anti-HIV activity.

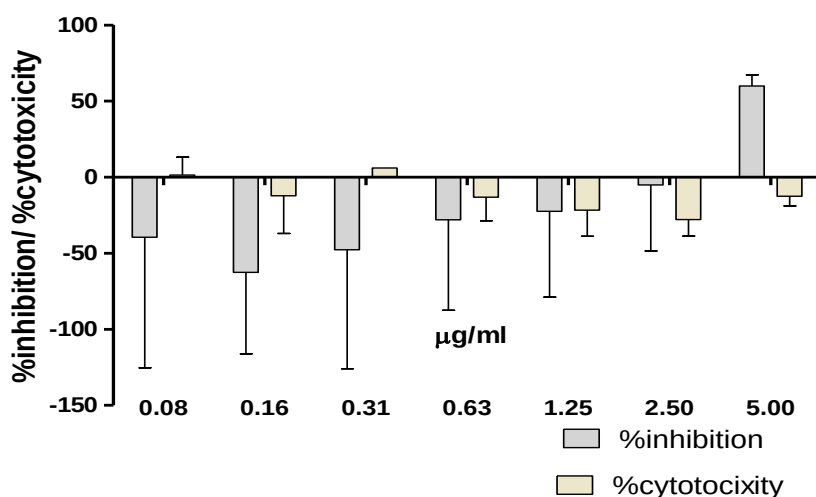


Figure 4.5: The in vitro anti-HIV activity of the *oven dried* 50% ethanol extract of the four plant mixture

4.6.4. *In vitro* latency reversal activity of *G. sericocephala* and the four plant mixture

The ethyl acetate fraction of *G. sericocephala* and the 50% ethanol extract of the four-plant mixture were also evaluated for their ability to activate HIV latent cell reservoirs in J-Lat 9.2 cells. The latency reactivation potential was measured by means of GFP expression in parallel with prostratin as a positive control. The ethyl acetate fraction and the 50% ethanol extract exhibited GFP expression in a dose dependent manner as shown in Figure 4.6.

The ethyl acetate fraction showed latency reversal at three different test concentrations. The fraction showed an increase in GFP expression of > 50% when tested at 1 µg/ml, > 75% when tested at 3 and 10 µg/ml. Moreover, in comparison to the positive control prostratin the ethyl acetate fraction showed comparable GFP expression at 3 and 10 µg/ml. These results also provided evidence that the ethyl acetate fraction did not lose its latency reversal activity during the scale-up process. However, the 50% ethanol extract of the four-plant mixture did not retain its latency activity as it only expressed GFP at the high test concentration (10 µg/ml) compared to the 50% ethanol extract of the four plant mixture obtained in lab scale with GFP expression at 1 µg/ml (Figure 2.31 Chapter 2). This could be attributed to the decomposition of the active compounds during the oven drying process, the active compounds were not extracted in sufficient quantities during scale up and dilution of the active compounds due to the extraction of the non-active compounds from the other plants. Further extraction optimization procedures in order to retain the activity of the four-plant mixture is required.

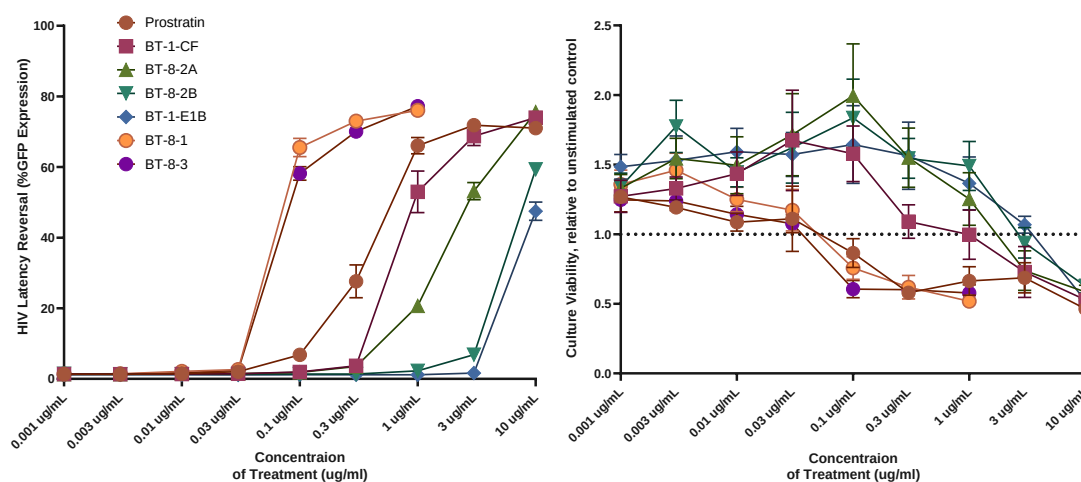


Figure 4.6: The HIV latency reversal activity of the ethyl acetate fraction of *G. sericocephala* (BT-1-CF) with an IC_{50} equivalent to 0.3 µg/ml and the 50% ethanol extract of the four plant mixture (BT-1-E1B) measure by GFP expression in J-Lat 9.2 cells with an IC_{50} 10 µg/ml. The samples labelled BT-8-2A, BT-8-2B, BT-8-1 and BT-8-3 do not form part of this chapter and were discussed in chapter 3.

The ethyl acetate fraction did not exhibit cytotoxicity in J-Lat 9.2 cells at the most active test concentrations (10, 3 and 1 µg/ml). The compounds (yuanhuacine A and the mixture of

yuanhuacine and cis 2''/3'' isomer of gnididin) were found to be the most active compounds in reversing latency reversal activity in reservoirs in J-Lat 9.2 cells (Chapter 3), these compounds were also detected in the ethyl acetate fraction of *G.sericocephala* roots (Chapter 2). The 50% ethanol extract also did not exhibit cytotoxicity at the high-test concentration which was the only test concentrations the extract expressed GFP.

4.6.5. UPLC-QTOF-MS analysis of *G. sericocephala* ethyl acetate fraction

The chemical profile of the semi-pilot scale ethyl acetate fraction in ESI positive mode is shown in Figure 4.7. The full chemical analysis of the active ethyl acetate fraction was carried out to provide the full chemical fingerprint of the fraction for quality control purposes. The profile showed the presence of peaks that eluted within the polar and nonpolar region. A total of 11 compounds were tentatively identified as shown in Table 4.4. The compounds belonged to a variety of chemical classes including the anti-HIV active daphnane diterpenoids. The presence of these compounds was detected within the region of 11 to 13 minutes of the chromatogram.

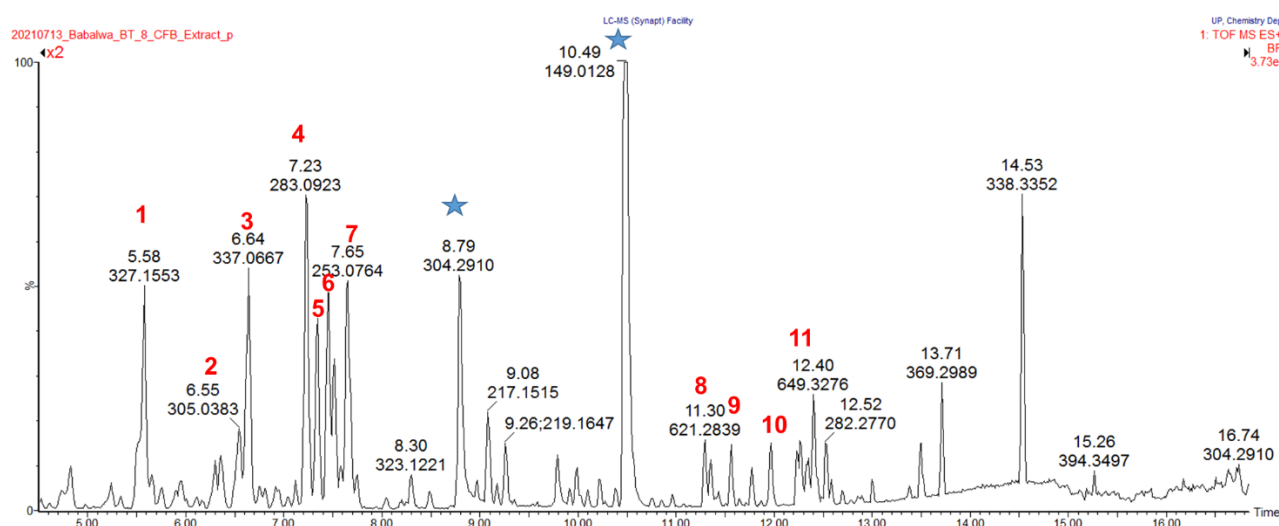


Figure 4.7: The chemical profile of the ethyl acetate fraction of *G. sericocephala* chromatogram in the ESI positive mode. The chromatogram shows a total of 11 compounds eluting between 5.58- 12.40 minutes. The profile also shows the presence of daphnane diterpenoids eluting between 11.30-12.40 minutes (blue star represents: peaks eluting at 8.79 and 10.49 minutes were also found in the blank.)

The $[M+H]^+$ ion observed for peak 1 at m/z 327.1553 corresponded to molecular formula $C_{20}H_{23}O_4$. The MS/MS spectra revealed the presence of a fragment ion at m/z 231.0753 suggesting that the molecule had experienced a cleavage on its aldehyde functional group in the aromatic moiety of the molecule, followed by a subsequent loss of the phenol ring to give fragment ion at m/z 160.9926. The molecule also experienced a retro Diels-Alder rearrangement to give fragment ion at m/z 137.0589. The compound was tentatively identified as dehydrodiisoeugenol. The $[M+H]^+$ ion observed for peak 2 at m/z 305.0495 corresponded to molecular formula $C_{18}H_{19}O_5$. The MS/MS spectra showed the most abundant ion (base peak) at m/z 221.0756 due to the loss of C_3H_2O and a consecutive loss of two water molecules. Based on the accurate mass, retention time, the fragmentation pattern and the NMR spectral data of the isolated lasioerin (chapter 3), peak 2 of the ethyl acetate fraction of *G. sericocephala* was identified as lasioerin. Additionally, the isolated lasioerin at m/z 305.0589 and peak 2 in the ethyl acetate fraction had the same retention time of 6.54 minutes as shown in Figure 4.8.

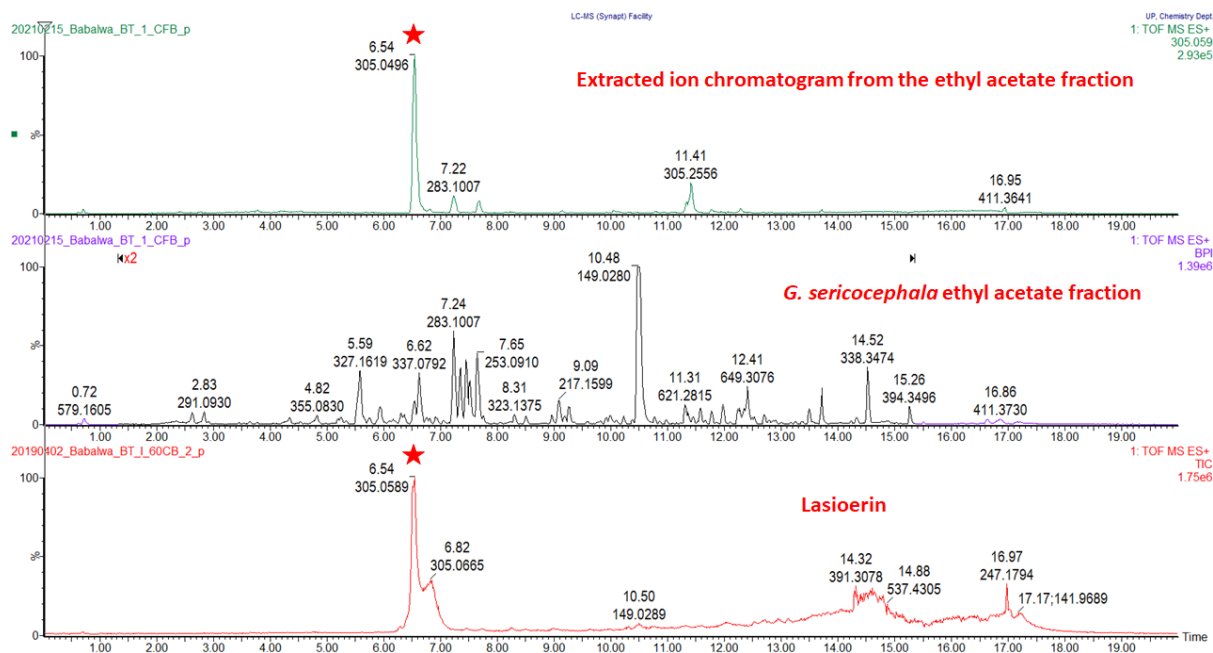


Figure 4.8: ESI positive mode BPI chromatogram of the ethyl acetate fraction of *G.sericocephala* and lasioerin pure standard. The pure standard had a retention time of 6.54 and an m/z 305.0589 and the peak 2 had a retention time of 6.54 minute and an m/z 305.0495.

The $[M+H]^+$ ion observed for peak 3 at m/z 337.0792 corresponded to molecular formula $C_{19}H_{13}O_6$. The MS/MS spectra revealed the presence of a fragment ion at m/z 321.0547 which represented an exposure to the McLafferty rearrangement and a fragment ion with a formula of $C_{18}H_9O_6$ was produced. The subsequent loss of two water molecules followed by a retro-Diels Alder mechanism to give fragment ion at m/z 237.0693. The molecule also experienced the loss of a water molecule to give fragment ion at m/z 221.0720. The fragmentation pattern led to the molecule being tentatively identified as lasiocephalin. A comparison of the accurate mass, retention time and the fragmentation pattern of peak 3 in the ethyl acetate fraction with the isolated lasiocephalin (Chapter 3) shown in Figure 4.9 confirmed the identity of peak 3 as lasiocephalin. However, there was a small difference in the retention time of lasiocephalin (6.68 min at m/z 337.0922) with that of peak 3 in the ethyl acetate fraction (6.62 min at m/z 337.0792). The difference in retention time in the fraction and lasiocephalin falls within the accepted value 0.5 min difference and thus peak 3 was confirmed as lasiocephalin.

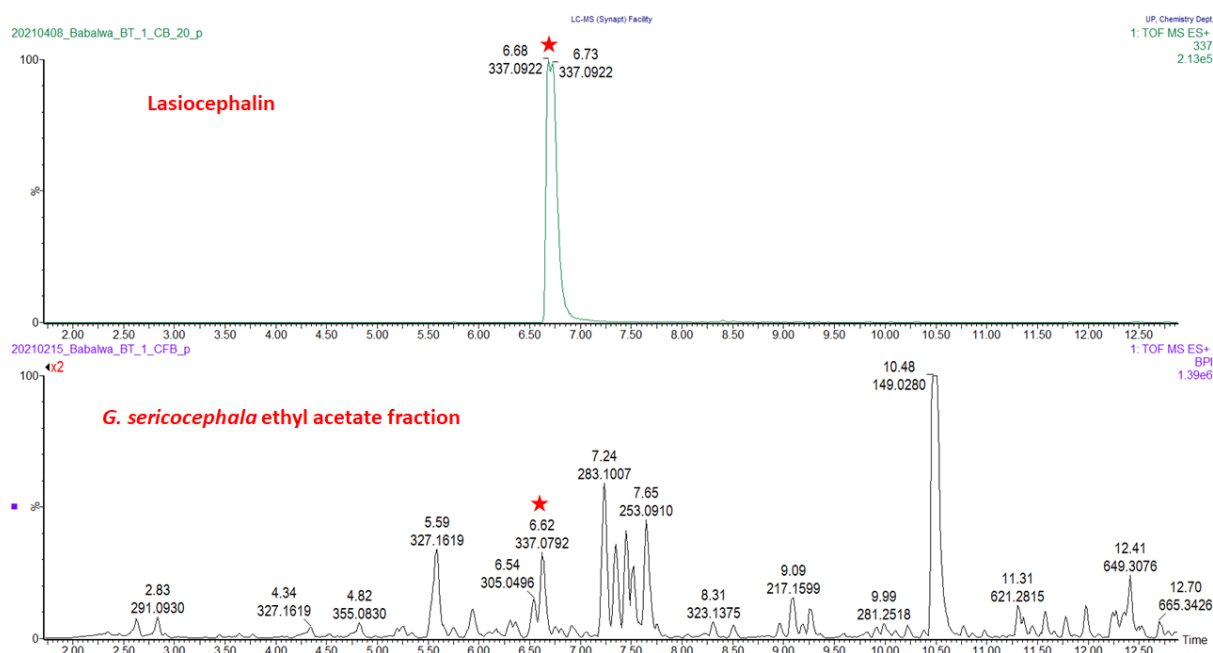


Figure 4.9: ESI positive mode BPI chromatogram of the ethyl acetate fraction of *G. sericocephala* and lasiocephalin pure standard. The pure standard had a retention time of 6.68 and an m/z 337.0922 and the peak 3 had a retention time of 6.68 minute and an m/z 337.0792.

The $[M+H]^+$ ion observed for peak 4 at m/z 283.0923 corresponded to molecular formula $C_{17}H_{14}O_4$. The MS/MS spectra revealed the presence of fragment ion at m/z 269.0813 $[M-12]$ due to a loss of a methyl and subsequently followed by a loss of a water molecule to give fragment ion at m/z 251.0692. The subsequent loss of a methoxy group revealed by the presence of the fragment ion at m/z 221.0614. The compound was tentatively identified as vadimezan. The $[M+H]^+$ ion observed for peak 5 at m/z 313.1028 corresponded to molecular formula $C_{18}H_{17}O_5$. The MS/MS spectra of the molecule revealed the presence of fragment ion at m/z 335.0201 $[M-Na]^+$ due to a sodium adduct. The molecule also experienced a McLafferty rearrangement and gave fragment ion at m/z 237.0552 $[M-C_6H_5]^+$ followed by a retro Diels-Alder rearrangement to give fragment ion at m/z 181.0654 which led to the tentative identification of the molecule being trimethoxyflavone. The $[M+H]^+$ observed for peak 7 at m/z 253.0835 corresponded to molecular formula $C_{16}H_{13}O_3$. The MS/MS spectra revealed the

presence of a fragmentation ion at m/z 238.0618 [M-15] due to the loss of a methyl group. The fragment ion at m/z 221.0602 was due to the loss of a methoxy group. A cleavage followed by a loss of an acetyl group was represented by fragment ion at m/z 210.0677. Based on this data the compound was tentatively identified as trimethoxyflavone. The [M+H]⁺ ion observed for peak 6 at m/z 223.0623 corresponded to molecular formula C₁₅H₁₁O₅. The compound was tentatively identified as isoflavone. The [M+H]⁺ ion observed for peak 7 at m/z 253.0910 corresponded to molecular formula C₁₆H₁₃O₃. The compound was tentatively identified as anisindione.

The [M+H]⁺ ion observed for peak 8 at m/z 621.2815 corresponded to molecular formula C₃₅H₄₁O₁₀. The MS/MS analysis gave a daphnane fragment at m/z 517.3700 due to the loss of a benzaldehyde moiety, this was also visible in the spectra at m/z 123.0805. This led to the compound being tentatively identified as yuanhuacine A. A comparison of the accurate mass, retention time and the fragmentation pattern of peak 8 in the ethyl acetate fraction of *G. sericocephala* roots and the isolated yuanhuacine A (chapter 3) is shown in Figure 4.10. A negligible difference in retention time of the isolated yuanhuacine A and peak 8 of 0.03 minutes was observed between the compound at m/z 621.2692 (11.28 min) and peak 8 at m/z 621.2670 (11.31 min) and the peak was identified as yuanhuacine A.

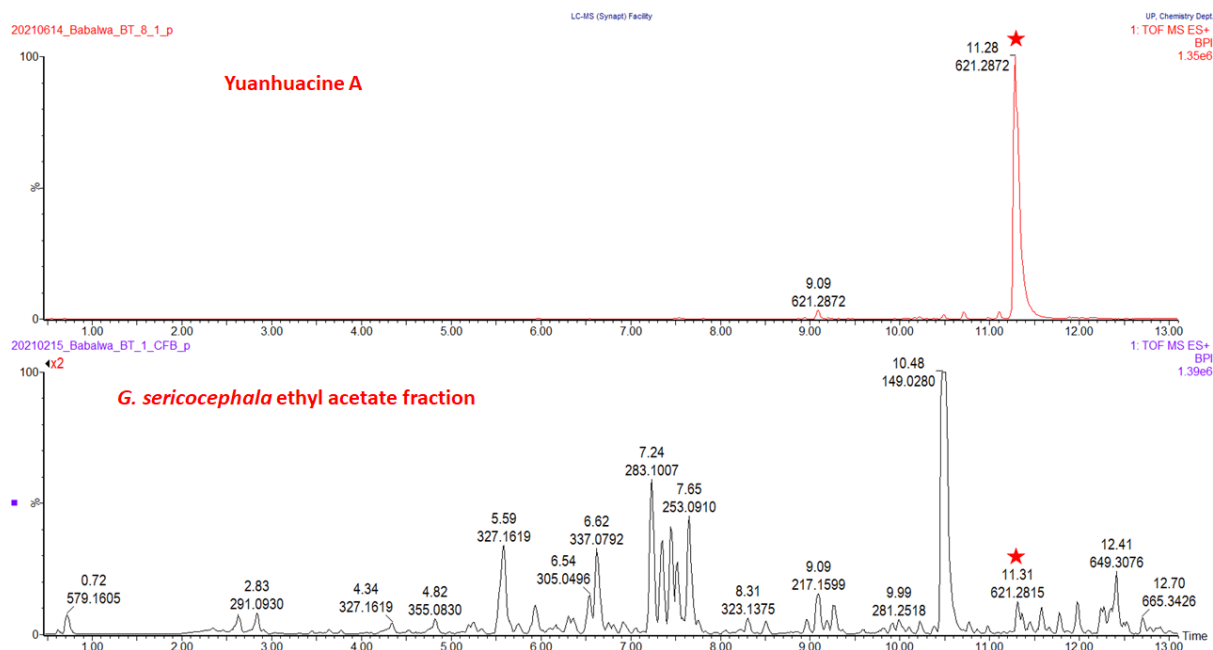


Figure 4.10: ESI positive mode BPI chromatogram of the ethyl acetate fraction of *G. sericocephala* and yuanhuacine A isolated as described in Chapter 3. The pure standard had a retention time of 11.28 and an m/z 621.2872 and the peak 8 had a retention time of 11.31 minute and an m/z 621.2815.

The $[M+H]^+$ ion observed for peak 9 at m/z 647.2947 corresponded to molecular formula $C_{37}H_{43}O_{10}$. The MS/MS spectra of the ethyl acetate fraction showed a sodium adduct ion at m/z 669.2689. The compound experienced a loss of its benzoic moiety shown by the presence of fragment ion at m/z 517.3845. The presence of the alkyl chain and the benzaldehyde functional groups was observed due to the presence of fragment ions at m/z 107.0534 and 165.0787, respectively. The fragmentation pattern led to the compound being tentatively identified as yuanhuajine. The comparison of the accurate mass, retention time and the fragmentation pattern of peak 9 and the isolated yuanhuajine (Chapter 3) is shown in Figure 4.11. A negligible difference in retention time of the isolated yuanhuajine and peak 9 of 0.02 minutes was observed between the compound at m/z 647.3044 (11.56 minutes) and peak 9 at m/z 647.2947 (11.58 minutes).

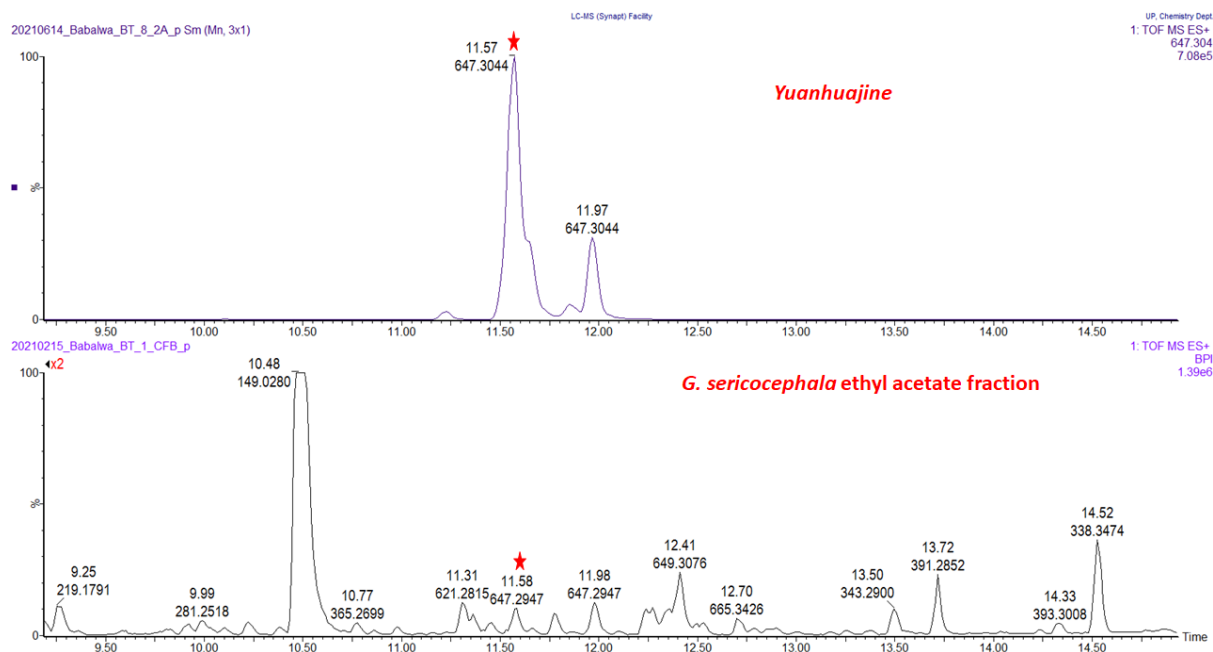


Figure 4.11: ESI positive mode BPI chromatogram of the ethyl acetate fraction of *G. sericocephala* and yuanhuajine pure standard. The pure standard had a retention time of 11.56 and an m/z 647.3044 and the peak 9 had a retention time of 11.58 minute and an m/z 647.2947.

The $[M+H]^+$ ion observed for peak 10 at m/z 647.2947 corresponded to molecular formula $C_{37}H_{43}O_{10}$. The compound was tentatively identified as gniditrin and further compared to the isolated gniditrin as shown in Figure 4.12. A negligible difference in retention time of the pure compound and peak 10 of 0.01 minutes was observed between the compound at m/z 647.3044 (11.97 min) and peak 9 at m/z 647.2947 (11.98 min) and the peak was identified as gniditrin.

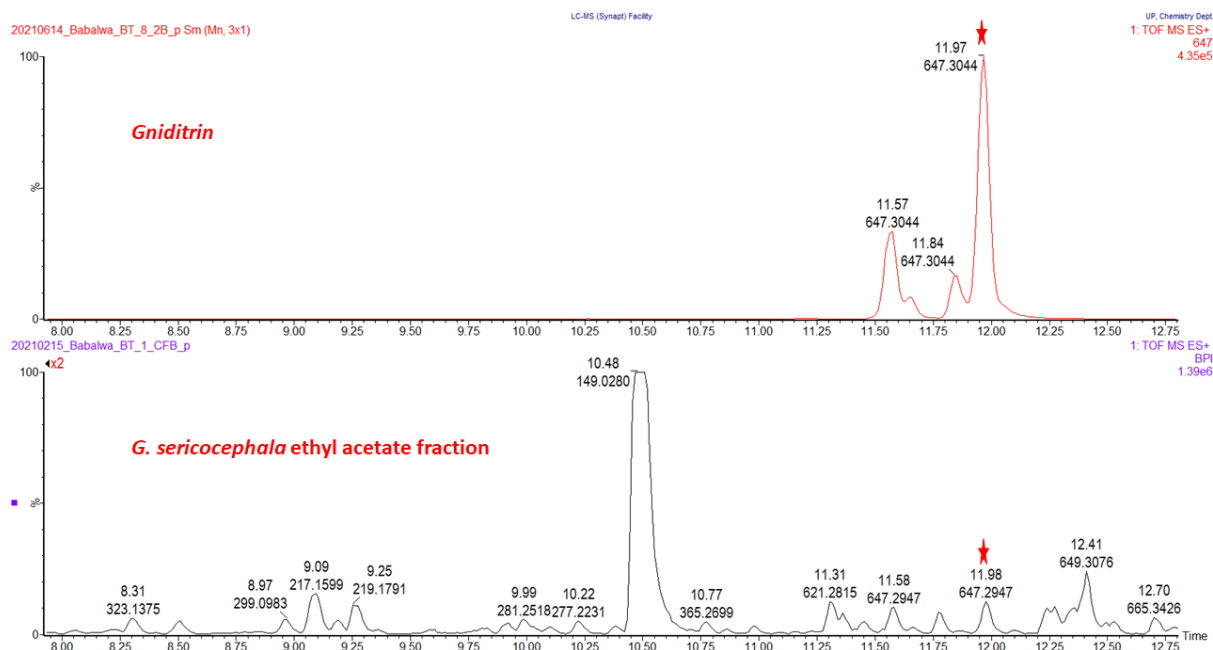


Figure 4.12: ESI positive mode BPI chromatogram of the ethyl acetate fraction of *G. sericocephala* and gniditrin pure standard. The pure standard had a retention time of 11.97 and an m/z 647.3044 and the peak 10 had a retention time of 11.98 minute and an m/z 647.2947.

The $[M+H]^+$ ion observed for peak 11 at m/z 649.3076 corresponded to molecular formula $C_{37}H_{45}O_{10}$. The MS/MS spectra showed of fragment ion $[M+Na]^+$ at m/z 671.2809. The presence of fragment ion observed at m/z 549.2434 was due the molecule undergoing a McLafferty rearrangement followed by the loss of its isopropenyl moiety to give fragment ion at m/z 503.1674. The isopropenyl moiety has been reported to be one of the daphnane diterpenoids characteristics.^[3,14,26–28] The spectra showed a fragment ion at m/z 381.1302 due to the loss of the alkyl chain. The comparison of the accurate mass, retention time and the fragmentation pattern of peak 11 and the isolated yuanhuacine as a mixture of isomers confirmed the compound. A comparison of peak 11 in the ethyl acetate fraction of *G. sericocephala* with that of the isolated yuanhuacine (Chapter 3) shown in Figure 4.13 also confirmed the identity of the peak. A negligible difference in retention time of yuanhuacine and peak 11 of 0.02 minutes was observed between the compound at m/z 649.3176 (12.39 min) and peak 11 at m/z 697.3076 (12.41 min) and the peak was identified as yuanhuacine as a mixture of isomers.

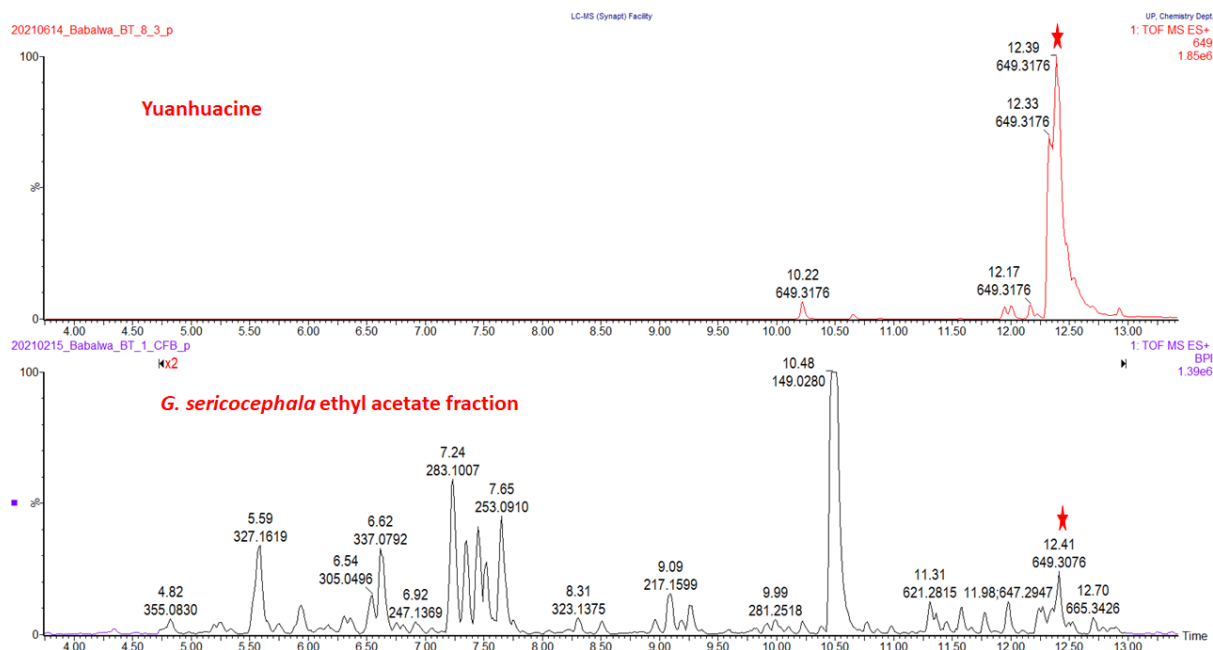


Figure 4.13: ESI positive mode BPI chromatogram of the ethyl acetate fraction of *G. sericocephala* and yuanhuacine. Yuanhuacine had a retention time of 12.39 and an m/z 649.3176 and the peak 11 had a retention time of 12.41 minute and an m/z 649.3076.

The presence of daphnane diterpenoids in the ethyl acetate fraction of *G. sericocephala* was successfully detected using UPLC-QTOF-MS in the chemical profile of this fraction. The chemical profile of this fraction can be further used as a reference for quality control purposes for future development of the traditional medicine. The most intense peaks in this fraction did not belong to the daphnane diterpenoid class. However, their presence is crucial in this product as they could aid the active compounds in being more bio-available. These results also support the retained anti-HIV activity and latency reversal activity shown by this fraction.

Table 4. 4: List of compounds identified from the ESI positive mode of the ethyl acetate fraction of *G. sericocephala* roots.

Peak No.	RT (min)	Observed [M+H] ⁺	Molecular Formula	Theoretical [M+H] ⁺	Mass error (ppm)	MS/MS Fragments	Tentative identification
1.	5.58	327.1553	C ₂₀ H ₂₃ O ₄	327.1590	-1.8	231.0753	dehydrodiisoeugenol
						160.9912	
						137.0686	
2.	6.54	305.0383	C ₁₈ H ₉ O ₅	305.0445	-1.6	249.0542	lasioerin
						221.0601	
						205.0658	
3.	6.62	337.0667	C ₁₉ H ₁₃ O ₆	337.0694	-5.3	265.0501	lasiocephalin
						221.0591	
						146.0368	
4.	7.24	283.0923	C ₁₇ H ₁₄ O ₄	283.0969	-0.4	267.0648	vadimezan
						239.0705	
						165.0703	
5.	7.34	313.1028	C ₁₈ H ₁₇ O ₅	313.1067	-2.9	297.0759	trimethoxyflavone
						283.0586	
						165.0179	
6.	7.45	223.0623	C ₁₅ H ₁₁ O ₂	223.0757	-0.9	129.0341	isoflavone
						77.0391	
7.	7.65	253.0764	C ₁₆ H ₁₃ O ₃	253.0866	0.4	210.0674	anisindione
8.	11.31	621.2839	C ₃₅ H ₄₁ O ₁₀	621.2682	-2.9	643.2479	yuanhuacine A
						621.2697	
						521.2092	
9.	11.57	647.3031	C ₃₇ H ₄₂ O ₁₀	647.2856	-4.4	669.2641	yuanhuajine
						647.2823	
						503.1670	
10.	11.98	647.3031	C ₃₇ H ₄₂ O ₁₀	647.2821	-3.4	669.2641	gniditrin
						647.2823	
						503.1670	
11.	12.41	649.2770	C ₃₇ H ₄₅ O ₁₀	649.2983	-4.6	671.2817	yuanhuacine
						649.2984	
						549.2433	

4.6.6. UPLC-QTOF-MS analysis of the four plant mixture extract

The UPLC-MS analysis of the 50% ethanol extract of the four plant mixture consisted of compounds that ionize in the positive ionization mode and compounds that ionize in the negative ionization mode. However, the active compounds from *G. sericocephala* were found to ionize better in the positive mode. In order to identify these daphnane diterpenoids in the mixture only the positive ionization mode of the extract is reported. The chemical analysis of

the 50% ethanol extract of the four plant mixture is shown for the top layer, bottom layer, which were dried using the Genevac, the dilute extract (extract initially received from Afriplex Pty Ltd) and the oven dried extract as shown in Figure 4.14.

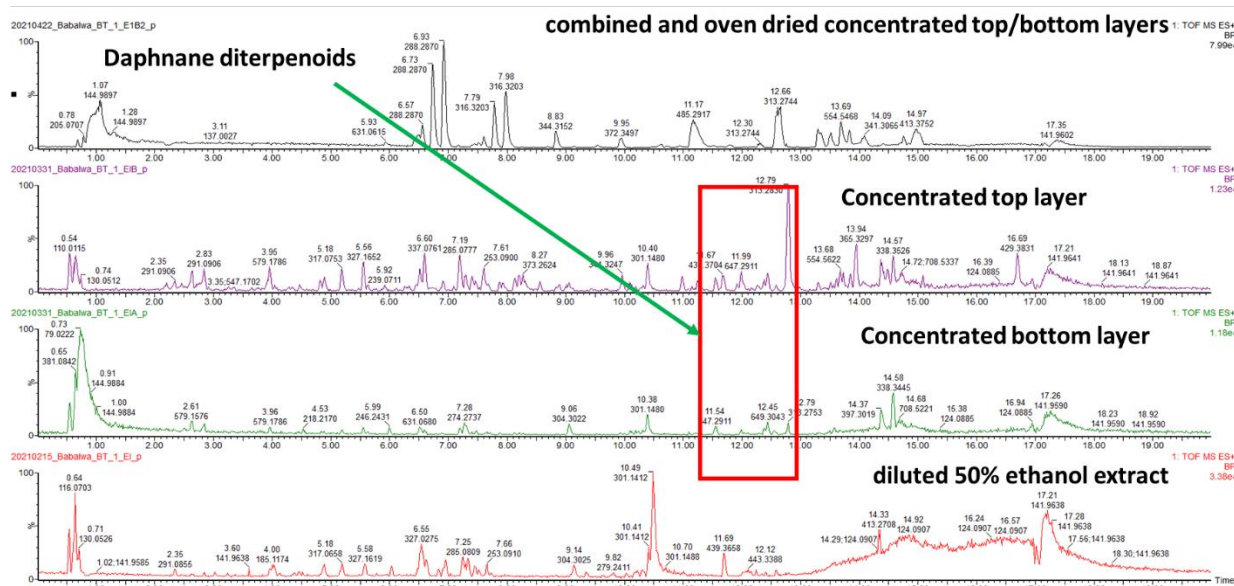


Figure 4. 14:The UPLC-QTOF-MS/ MS chemical profile of the combined and oven dried concentrated top/bottom layers, concentrated bottom layer, concentrated top layer and dilute 50% ethanol extract in the ESI positive mode. The concentrated top and bottom layer chromatograms showed a presence of daphnane diterpenoids.

The daphnane rich region (11.24- 12.45 min) of the chromatogram is expanded to get a clearer view of the daphnane diterpenoids present in the different extracts as shown in Figure 4.15. The diluted 50% ethanol extract was the initial extract obtained from Afriplex Pty Ltd. This extract showed the presence of only one daphnane diterpenoid eluting at 12.41 min with m/z 649.3556, the compound which was previously identified as yuanhuacine as a mixture of isomers until these were separated. The compound was also proven to be one of the responsible compounds playing a role in the anti-HIV and latency reversal activity in the plant *Gnidia*. After further concentrating the extract, the more concentrated extract having two layers namely the top layer and the bottom layer were also analysed separately for their chemical

profile. The chemical profile of the extracts both showed the presence of four daphnane diterpenoids which were isolated in this study, namely yuanhuacine A, yuanhuacine, yuanhuajine and gniditrin and the presence of these compounds indicated that the extraction process was a success. However, due to the nature of the extract, it needed to be dried further. Both layers were combined and dried in an oven however the chemical profile using UPLC-QTOF-MS did not show the presence of the daphnane diterpenoids. The absence of these compounds in the oven dried extract was an indication that a new drying method that will not lead to decomposition of active compounds is required. Additionally, it is possible that these compounds are not stable when exposed to higher temperatures. The anti-HIV and latency reversal activity results of the oven dried extract of the four plant mixture when tested at high test concentrations indicated.

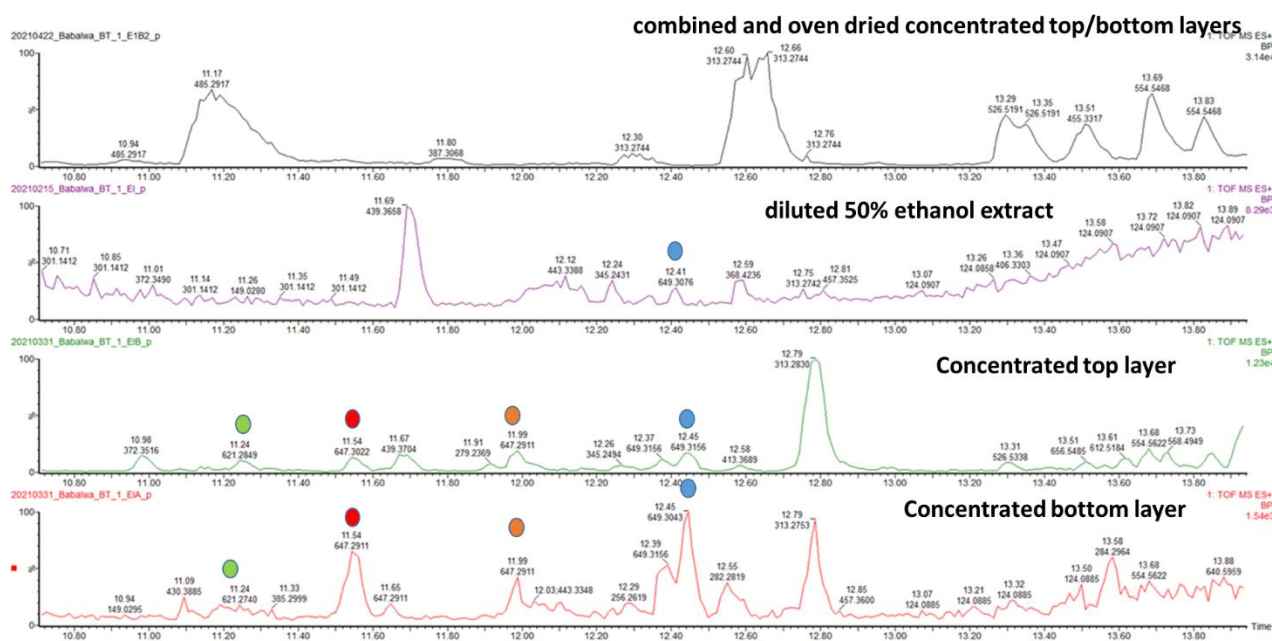


Figure 4. 15: The expanded UPLC-QTOF-MS/ MS chemical profile of the combined and oven dried concentrated top/bottom layers, concentrated bottom layer, concentrated top layer and dilute 50% ethanol extract in the ESI positive mode. The concentrated top and bottom layer chromatograms showed a presence of daphnane diterpenoids. (green) yuanhuacine A; (red) gniditrin; (orange) yuanhuajine; and (blue) yuanhuacine as a mixture of isomers. While the diluted 50% ethanol extract showed the presence of one daphnane diterpenoid.

The initially prepared dilute extract obtained from Afriplex Pty Ltd showed the presence of only one daphnane diterpenoids with m/z 649.3076 which was previously identified as yuanhuacine (see Chapter 3, section 3.6.13.3.). The top and bottom layer after concentrating both showed the presence of daphnane diterpenoids identified as yuanhuacine A (m/z 621.2740), yuanhuajine and gniditrin (m/z 647.3911) and yuanhuacine (m/z 649.1245). After oven drying the compounds were no longer not detected in the mixture, suggesting that the compounds were sensitive to heat. This can imply that the effectiveness of the extract is due to the presence of daphnane diterpenoids. However, the activity that is seen at the high test concentration could be due to another non-daphnane diterpenoid compound or the synergistic effect of the compounds in the mixture. There is a need for future work on the drying or development of new extraction optimization procedures for the development of the ethanol extract of the four-plant mixture in order to retain the presence of daphnane diterpenoids in the mixture.

4.6.7. Quantification of the yuanhuacine A in the ethyl acetate fraction of *G.sericocephala*

Five different concentrations of yuanhuacine A between a concentration range of 1 mg/L to 20 mg/L were prepared, with triplicate injections for each concentration on the UPLC-QTOF-MS. The calibration curve shown in Figure 4.16. was determined by plotting the peak areas of yuanhuacine A versus their respective concentrations. The coefficient of correlation (R^2) was obtained as 0.9999 this value is greater than the ICH ($R^2 = 0.90$) acceptable value. The precision and repeatability of the method expressed as mean concentration and %RSD were within acceptable limits as the %RSD was below the acceptable limit of less than 5% as shown Table 4.5.

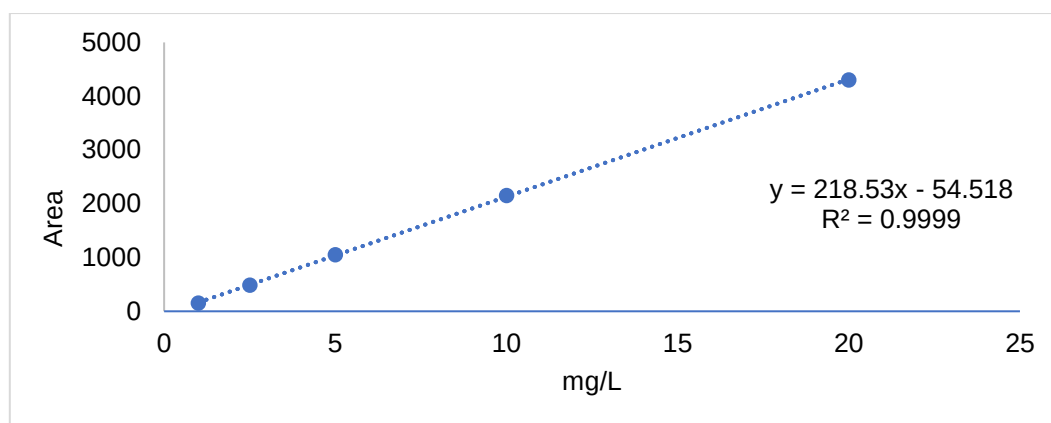


Figure 4.16: Yuanhuacine A calibration curve obtained at 5 different concentration points with a coefficient of correlation $R^2 = 0.9999$.

Table 4.5: Intra-day precision of the UPLC-MS method for yuanhuacine A

Concentration mg/L	Mean area under the curve	%RSD
1	135.994	3.96
2.5	620.231	0.46
5	1312.390	0.53
10	2360.577	2.26
20	5289.08	0.16

The LoD and LoQ for yuanhuacine A as shown in Table 4.6 were calculated using regression analysis and were obtained as 0.02 mg/L and 0.13 mg/L, respectively, suggesting a good sensitivity of the method used.

Table 4. 5: Quantification parameters of yuanhuacine A from the ethyl acetate fraction of *G. sericocephala*

Compound	Linear range	Regression analysis	R^2	LoD (mg/L)	LoQ (mg/L)
Yuanhuacine A	1-20 mg/L	$Y = 218.53x - 54.518$	0.9999	0.02	0.13

The concentration of yuanhuacine A in the ethyl acetate fraction of *G. sericocephala* (injected as a 1000 mg/L solution), was determined using the regression analysis equation (Table 4.6) and determined as 15.176 mg/L based on the area under the peak of 3262 after integration. Based on these results the dry ethyl acetate fraction contained 1.5% w/w of yuanhuacine A. These results show that there is sufficient quantities of the compound in the fraction which is based on the IC_{50} 0.03 μ M of the compound when screened against HIV in PBMC's and its ability to express HIV Latency reversal activity at 0.15 μ M as shown in Chapter 3 (Figure 3.82 and 3.84, respectively).

4.7 Conclusion

The study reported for the first time the semi-pilot scale development of the ethyl acetate fraction of *G. sericocephala* roots using GMP guidelines outlined for the development of traditional Medicine. The developed product passed all quality control analysis requirements which included physical, chemical, heavy metal, total solids, aflatoxins, microbial, and pesticides specifications. The fraction also showed good anti-HIV and latency reversal activity with retained *in-vitro* (IC_{50} = 0.05 μ g/ml) , *ex-vivo* (IC_{50} = 0.06 μ g/ml) anti-HIV as well as the *in vitro* (IC_{50} = 0.3 μ g/ml) latency reversal activity of the fraction. This retained activity also suggested that the extraction in pilot scale production was a success.

The UPLC-MS profile of the ethyl acetate fraction revealed a presence of 11 compounds, of which four belonged to the class of daphnane diterpenoids and were isolated for the first time from *G. sericocephala* roots in this study and identified as yuanhuacine A, yuanhuajine, gniditrin and yuanhuacine . These compounds (the daphnane diterpenoids) were proven to be responsible for the anti-HIV and latency reversal activity. The latency reversal activity of the ethyl acetate fraction of *G.sericocephala* was found to be comparable to prostratin. The quantitative analysis of yuanhuacine A in the ethyl acetate fraction of *G. sericocephala* using a five-point concentration in triplicate suggested that the fraction constitutes of 1.5 % of

yuanhuacine A in the ethyl acetate fraction. The ethyl acetate fraction was found to contain an effective quantities of yuanhuacine A.

The 50% ethanol extract of the four plant mixture was also developed for the first time in a pilot scale following GMP guidelines. The extract passed all quality control specifications such as physical, chemical, heavy metal, aflatoxins, pesticides, total solids, and microbial specifications. The semi-pilot scale extraction of the 50% ethanol of the four plant mixture however, posed some challenges as more advanced drying techniques were required to completely remove the solvents in the extract. Before the extract was exposed to high temperatures its chemical fingerprint using UPLC-MS showed the presence of daphnane diterpenoids, however after the extract was exposed to high temperatures during oven drying the daphnane diterpenoids were not detected. The absence of daphnane diterpenoids in the extract resulted to a larger extent to the loss of the anti-HIV and latency reversal activity of the extract in lower test concentration when compared to the laboratory developed product. seen from the in the bioactivity of the 50% ethanol extract of the four-plant mixture. The oven drying process has been shown to not be an ideal method of drying these products, a more suitable drying methods that requires lower temperatures to avoid the loss of the active ingredient is required for this extract. Due to this, yuanhuacine A could not be quantified in the oven dried extract.

4.8 References

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Chapter 5

Summary

This project forms part of a research based on claims made by a South African traditional health practitioner Nkabinde on using a traditional medicine consisting of a mixture of four plants, for the treatment of HIV (The documented information provided to the University of Pretoria). The plants, *S. birrea* stems (126589), *G. sericocephala* roots (126590), *S. italica* roots (126591) and *P. prunelloides* roots (126592) were collected from Tugela Ferry in Msinga region of KwaZulu Natal and delivered by THP Nkabinde, who disclosed the traditional use of the plants to the University of Pretoria. The plants were identified at the H.G.W.J Schweickerdt Plant Herbarium at the University of Pretoria with their respective voucher specimen numbers.

The dichloromethane : methanol extracts of each plant and the four plant mixture were prepared . This solvent system was chosen as it extracts both polar and non-polar compounds. All extracts were tested for their anti-HIV activity using a pNL-NF plasmid in a DeCiPhR assay at a dual concentration of 2.5 and 0.25 µg/ml with efavirenz as a positive control. The cytotoxicity of the extracts was also determined.

The DCM : MeOH extract of *S. birrea* leaf inhibited the virus at 25 µg/ml only, while the stem bark of *S. birrea* was inactive. The DCM : MeOH extract of *G. sericocephala* showed very good inhibition activities at 25 and 2.5 µg/ml with observed cytotoxicity levels. The DCM : MeOH extract of the mixture exhibited good anti-HIV activity mostly at 25 µg/ml and was attributed to the presence of the *G. sericocephala*, and the synergistic effect of the compounds present in the four plants extracts. Further screening of the dichloromethane: methanol extract of the mixture and *G. sericocephala* showed significant anti-HIV activity that is dose dependant.

In order to understand the HIV inhibitory activity of *G. sericocephala* the plant was extracted sequentially using hexane, dichloromethane, ethyl acetate and methanol. All the extracts were investigated in a DeCiPhR assay in a concentration-response manner. The dichloromethane

extract of *G. sericocephala* gave the best anti-HIV activity as it was the only extract to exhibit good anti-HIV activity (>80%) in lower doses (at 0.08 µg/mL). The dichloromethane extract was then investigated further for its anti-HIV properties to determine compounds responsible for the HIV inhibitory activity.

The roots of *G. sericocephala* were then extracted with pharmaceutically accepted solvents, in order to produce a standardized herbal medicine for the treatment of HIV. The ethanol extract of *G. sericocephala* and 50% ethanol of the four plant mixture gave the best anti-HIV activity. The anti-HIV activity in the ethyl ethanol extract was increased by partitioning the extract between ethyl acetate and water. The ethyl acetate fraction showed excellent anti-HIV activity as well as latency reversal activity. The 50% ethanol extract of the four plant mixture gave the best anti-HIV and latency reversal activity.

The chemical profiles (50% ethanol extracts) of the four plants will aid in the full characterization of the four plant mixture. Additionally, the chemical profile of the four plants will be used for quality control in the further developing the four plant mixture 50% ethanol extract. The UPLC-MS profile of the ethyl acetate fraction compared to the ethanol extract of *G. sericocephala* showed a decrease in peak intensity of the inactive compounds. The dichloromethane extract was further analysed using UPLC-QTOF-MS to identify compounds that exhibit HIV inhibition activities. The presence of daphnane diterpenoids in the extracts of *G. sericocephala* supported the observed anti-HIV properties possessed by this plant.

Different chromatographic techniques were used to fractionate and purify the DCM extract of *G. sericocephala* with the aim to obtain single pure active compounds with HIV inhibitory activity and HIV latency reversal activity. The study reported for the first time diterpenoids present in *G. sericocephala* roots based on the traditional mixture used in the treatment of HIV. The purification of these compounds was made successful by following multiple purification steps. These include the HPLC microfractionation which guided the purification process, as it localised the activity leading to microfractions 25, 26, 27, 28 and 29 being the

only fractions that inhibited activity of the pNL-NF plasmid f HIV in 293T cells by $52.0 \pm 0.9\%$, $71.0 \pm 5.9\%$, $83.9 \pm 9.6\%$, $103 \pm 2.1\%$, $101 \pm 2.0\%$ and $91.6 \pm 2.8\%$, respectively, from a total of 30 microfractions. The chemical analysis of the active microfractions using UPLC-QTOF-MS/MS revealed the presence of daphnane diterpenoids which were further purified by repetitive chromatographic techniques.

The anti-HIV activity in the DCM extract was successfully localised by HPLC directed fractionation. The UPLC-QTOF-MS/MS profile of the combined microfractions showed the presence of coumarins and daphnane diterpenoids, of which the latter was latter is known for its anti-HIV activity. The daphnane diterpenoids corresponding to m/z 621.2669 (peak 1), m/z 647.2833 (peak 2 and 3), and m/z 649.3020 (peak 4) were tentatively identified yuanhuacine A, yuanhuajine, gniditrin and yuanhuacine. This was based on the class of these compounds (daphnane diterpenoids) being known for having potent anti-HIV properties. These compounds were hypothesised to be responsible for the observed anti-HIV activity in the microfractions and were prioritised for isolation and purification. The UPLC-QTOF-MS/MS of the microfractions also revealed that the compounds were medium to non-polar based on their retention time. The inactive major peaks in the extract were successfully isolated as biomarkers and later identified using 1D and 2D NMR as lasioerin and lasiocephalin.

The preparative purification of the active fractions resulted in the isolation of five daphnane diterpenoids (yuanhuacine A, yuanhuacine, cis 2''/3'' isomer of gnididin, yuanhuajine and gniditrin) with viral inhibition properties as well as latency reversal activity. The compound yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin showed *in vitro* HIV inhibition of the pNL-NF-plasmid of HIV in 293T cells by $> 80\%$ at $0.08 \mu\text{g/ml}$. Furthermore, yuanhuacine A inhibited a subtype C virus replication in PBMC with an EC_{50} of $0.03 \mu\text{M}$ without cytotoxicity. The compound yuanhuacine A, yuanhuacine and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin also reverse HIV latency *in vitro* 16.7-fold lower concentrations than control prostratin. The compounds (yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin) also reverse latency in primary CD4⁺ T cells from cART-suppressed

donors with HIV similar to prostratin but at 6.7-fold lower concentrations. These results suggests that *G. sericocephala*, yuanhuacine A and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin act as anti-HIV agents to improve cART efficacy and support HIV cure efforts in resource-limited regions.

The study reported for the first time the semi-pilot scale development of the ethyl acetate fraction of *G. sericocephala* roots using GMP guidelines outlined for the development of traditional Medicine. The developed product passed all quality control analysis requirements which included physical, chemical, heavy metal, total solids, aflatoxins, microbial, and pesticides specifications. The fraction also showed activity against the pNL-NF plasmid of the HIV and latency reversal activity with an $IC_{50} = 0.05 \mu\text{g/ml}$, and anti-HIV activity in an *ex-vivo* model with an $IC_{50} = 0.06 \mu\text{g/ml}$. The fraction also showed good *in vitro* HIV latency reversal with an $IC_{50} = 0.3 \mu\text{g/ml}$. This retained anti-HIV and HIV latency reversal activity by the fraction suggested that the extraction in semi-pilot scale production was a success.

The UPLC-qToF-MS/MS profile of the ethyl acetate fraction revealed a presence of 11 compounds, of which four belonged to the class of daphnane diterpenoids and were isolated for the first time from *G. sericocephala* roots in this study and identified as yuanhuacine A, yuanhuajine, gniditrin and the mixture of yuanhuacine and cis 2''/3'' isomer of gnididin. These compounds (the daphnane diterpenoids) were proven to be responsible for the anti-HIV and latency reversal activity. The latency reversal activity of the ethyl acetate fraction of *G. sericocephala* was found to be comparable to prostratin. The quantitative analysis of yuanhuacine A in the ethyl acetate fraction of *G. sericocephala* using a five-point concentration in triplicate suggested that the fraction constitutes of 1.5 % of yuanhuacine A in the ethyl acetate fraction.

The 50% ethanol extract of the four plant mixture was also developed for the first time in a pilot scale following GMP guidelines. The extract passed all quality control specifications such as physical, chemical, heavy metal, aflatoxins, pesticides, total solids, and microbial specifications. The semi-pilot scale extraction of the 50% ethanol of the four plant mixture

however, posed some challenges as more advanced drying techniques were required to completely remove the solvents in the extract. Before the extract was exposed to high temperatures its chemical fingerprint using UPLC-qToF-MS/MS showed the presence of daphnane diterpenoids. However, after the extract was exposed to high temperatures during oven drying the daphnane diterpenoids were not detected. The absence of daphnane diterpenoids in the extract resulted to a larger extent to the loss of the anti-HIV and latency reversal activity of the extract in lower test concentration when compared to the laboratory developed product. seen from the in the bioactivity of the 50% ethanol extract of the four-plant mixture. The oven drying process has been shown to not be an ideal method. A more suitable drying methods that requires lower temperatures to avoid the loss of the active ingredient is required for this extract. Due to this, yuanhuacine A could not be quantified in the over dried extract.

Moreover, the dual activity inhibition of HIV replication and latency reversal shown by these compounds suggests a further investigation on the mechanisms of this plant in additional cell and animal models. As South Africa continues to harbour a high number of people living with HIV that are resistant to available antiretroviral therapy with limited access to existing cART, *G. sericocephala* root extracts and its compounds, could act as useful leads toward additional therapies for HIV/AIDS management to supplement cART in South Africa.

Supplementary data

The extracts and compounds of *G. sericocephala* were investigated for their ability to re-verse HIV latency using J-Lat T cells (clone 10.6) obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (contributed by Dr. Eric Verdin) (Jordan et al., 2003). The cells were cultured R10+ media [RPMI 1640 with HEPES and L-Glutamine, 10% Fetal Bovine Serum (FBS), 100 U of penicillin/mL, and 100 µg of streptomycin/mL (Sig-ma-Aldrich, St. Louis, MO, USA) and incubated at 37 °C and 5% CO₂. Prostratin and Gö 6983 were purchased from Sigma-Aldrich. Enzastaurin was purchased from Sellick Chemicals GmbH through ThermoFisher (Waltham, MA, USA).

The cells were seeded into 96-well plates at 2 x 10⁵ cells/well in the presence of test agents at defined concentrations or 0.1% DMSO vehicle control and incubated as described above for 24 hours. GFP expression in J-Lat 10.6 cells was monitored by flow cytometry using a FACSCelesta analyzer (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using FlowJo v. 10.5.3 (FlowJo LLC; Ashland, OR, USA). All presented results represent the mean ± S.D. from 3 independent experiments.

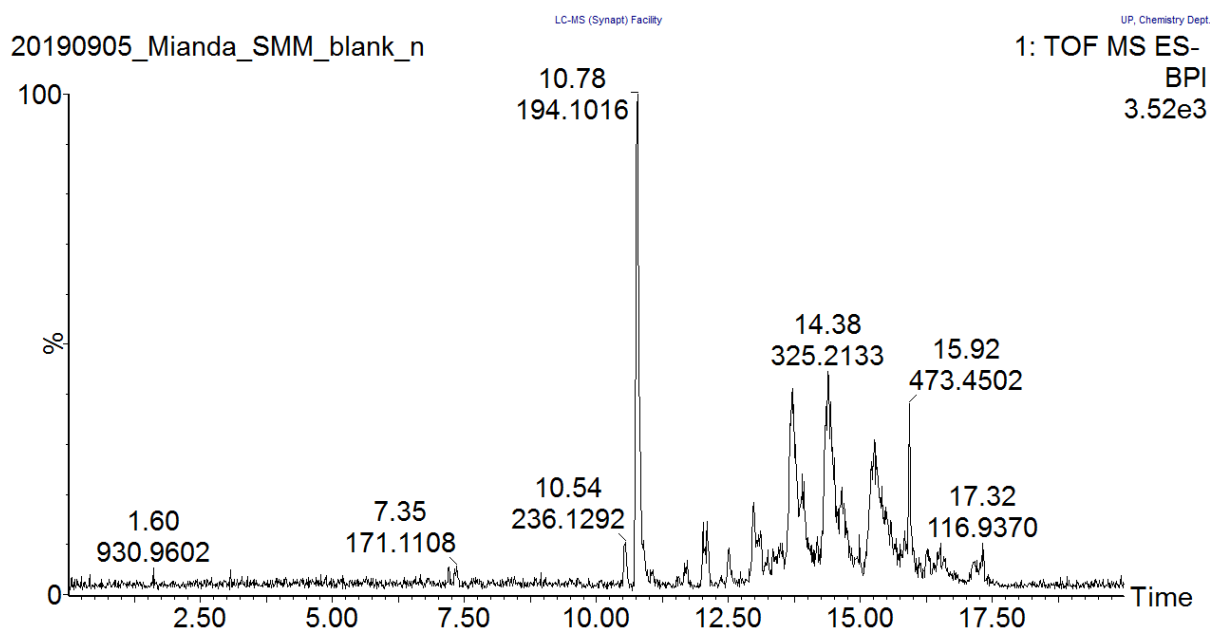
To measure cell viability, Jurkat T cells (Clone E6-1) obtained from the American Tissue Culture Collection (Manassas, VA, USA) were treated as described above before addition of resazurin (Sigma-Aldrich) to a final concentration of 20 µg/mL. Cells were then incubated for an additional 4 hours before fluorescence intensity was measured using a ClarioStar plate reader (BMG Labtech). Background fluorescence was subtracted from wells containing resazurin and R10+ medium but no cells. To measure T cell activation, Jurkat cells were treated as described above before staining with CD69-phytoerythrin (BD Biosciences; Franklin Lakes, NJ, USA) antibody using the Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions. Flow cytometry was then performed as described above.

Ex vivo latency reversal assays

The compounds were tested for ability to reverse HIV latency using primary cell isolates. Primary CD4⁺ T cells were obtained from three HIV-1 infected donors on stably suppressive cART (< 50 copies/mL of plasma viral load) for at least 3 years following informed written consent. These participants were recruited in accordance with human subject research guidelines of the United States Department of Health and Human Services under the supervision of the Wistar and Philadelphia FIGHT institutional review boards. CD4⁺ T cells were isolated from PBMC using the EasySep Human CD4⁺ T cell Enrichment Kit (STEM-CELL, Vancouver, Canada) and cultured in R20⁺ media, which was identical to R10⁺ media described above except for addition of 20% FBS.

Primary cell viability and latency reversal in the presence of test agents were performed as described previously (Schonhofer et al., 2021). Briefly, 2 x 10⁶ cells were treated with 0.1% DMSO, test agent, or anti-CD3/CD28 dynabeads (Invitrogen, Carlsbad, CA, USA) in a 1:1 ratio for 24 hours. Following incubation, live cells were counted visually by trypan blue staining. Viral RNA was extracted from culture supernatants using a QIAmp Viral RNA Mini kit (Qiagen, Germantown, MD, USA) and subjected to quantitative RT-PCR as described previously using a C1000 Thermal Cycler and CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA) (Yuan et al., 2017). The limit of detection of super-natant viral RNA was 10 copies/mL as determined through repeating end point detection from serial dilution of the AcroMetrix HIV-1 Panel (ThermoFisher).

Supplementary data 1: Latency reversal activity of *G. sericocephala* and its compounds.



Supplementary data 2: UPLC-MS chromatogram of the solvent blank

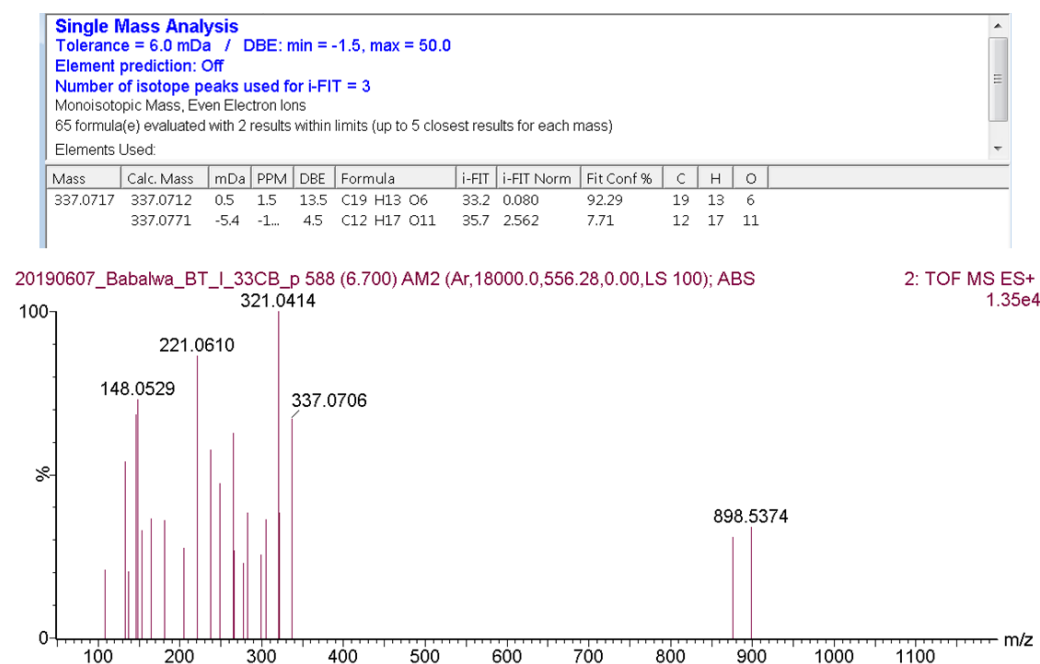
Anti-HIV Efficacy Evaluation in Fresh Human PBMCs Fresh human PBMCs, seronegative for HIV and HBV, are isolated from screened donors (New York Blood Center). Cells are pelleted/washed 2-3 times by low speed centrifugation and re-suspended in PBS to remove contaminating platelets. The Leukophoresed blood is then diluted 1:1 with Dulbecco's Phosphate Buffered Saline (DPBS) and layered over 15 mL of Ficoll (GE Healthcare #17-1440-02) in 50 mL centrifuge tube and then centrifuged for 30 minutes at 600 X g. Banded PBMCs are gently aspirated from the resulting interface and subsequently washed 2X with PBS by low speed centrifugation. After the final wash, cells are enumerated by trypan blue exclusion and re-suspended at 1×10^6 cells/mL in RPMI 1640 supplemented with 15 % Fetal Bovine Serum (FBS), and 2 mM L-glutamine, 0.5 μ g/mL Phytohemagglutinin (PHA, Remel or comparable). The cells are stimulated in this medium for 48-72 hours at 37°C. After incubation, PBMCs are centrifuged and re-suspended in RPMI 1640 with 15% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 20 U/mL recombinant human IL-2 (R&D Systems, Inc). IL-2 is included in the culture medium to maintain the cell division initiated by the PHA mitogenic stimulation. MDMs are depleted from the culture as the result of adherence to the tissue culture flask. For the standard PBMC assay, PHA

stimulated cells from at least two normal donors are pooled (mixed together), diluted in fresh medium to a final concentration of 1×10^6 cells/mL, and plated in the interior wells of a 96 well round bottom microplate at 50 μ L/well (5×10^4 cells/well) in a standard format developed by the Infectious Disease Research department of Southern Research Institute. Pooling (mixing) of mononuclear cells from more than one donor is used to minimize the variability observed between individual donors, which results from quantitative and qualitative differences in HIV infection and overall response to the PHA and IL-2 of primary lymphocyte populations. Each plate contains virus/cell control wells (cells plus virus), experimental wells (drug plus cells plus virus) and compound control wells (drug plus media without cells, necessary for MTS monitoring of cytotoxicity). In this in vitro assay, PBMC viability remains high throughout the duration of the incubation period. Therefore, infected wells can be used in the assessment of both antiviral activity and cytotoxicity. Evaluations of cytotoxicity can also be optionally performed on uninfected cells. Test drug dilutions are typically prepared at a 2X concentration in microtiter tubes and 100 μ L of each concentration (nine total concentrations) are placed in appropriate wells using the standard format. 50 μ L of a predetermined dilution of virus stock is placed in each test well (final MOI $\cong 0.1$). The PBMC cultures are maintained for sixseven days following infection at 37°C, 5% CO₂. After this period, cell-free supernatant samples are collected for analysis of reverse transcriptase activity and/or p24 antigen content. Following removal of supernatant samples, compound cytotoxicity is measured by addition of MTS to the plates for determination of cell viability, if applicable. Wells are also examined microscopically and any abnormalities are noted. MTS staining for PBMC viability to measure cytotoxicity At assay termination, assay plates are stained with the soluble tetrazolium-based dye MTS (CellTiter 96 Reagent, Promega) to determine cell viability and quantify compound toxicity. At termination of the assay,

Supplementary data 3: Ex vivo anti-HIV activity in PBMC's

Concentration ($\mu\text{g/ml}$)	yuanhuacine A			<i>G. sericocephala</i> ethyl acetate			verapamil		
	$\bar{x}$	S.D	N	$\bar{x}$	S.D	N	$\bar{x}$	S.D	Acceptable range
0.02	3.19	5.53	3	4.17	3.63	3	0.61	0.11	02-08
0.2	16.65	8.37	3	8.82	5.61	3			
2	18.41	4.64	3	14.14	9.34	3			
20	32.29	1.20	3	25.69	11.09	3			
hERG potency (μM)	>20% $\mu\text{g/ml}$	-	3	>20% $\mu\text{g/ml}$	-	3			02-08

Supplementary data 4: Summary of mean potency values ($\text{IC}_{50} \pm \text{S.D.}$), inhibition values at each test concentration, for the test samples, the positive control, verapamil, potency. All data in tables are expressed as mean inhibition or mean μM potency, from $N \geq 3$ separate cells. The positive control value was within the acceptable potency range (0.2 - 0.8 μM).



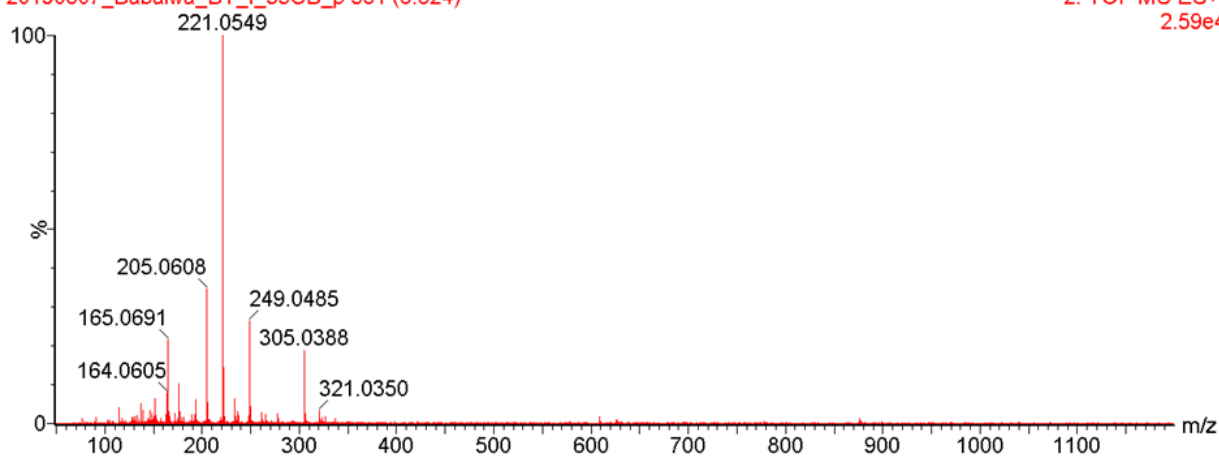
Supplementary data 5: MS/MS spectral data of lasioerin.

Single Mass Analysis
Tolerance = 6.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
56 formula(e) evaluated with 2 results within limits (up to 5 closest results for each mass)
Elements Used:

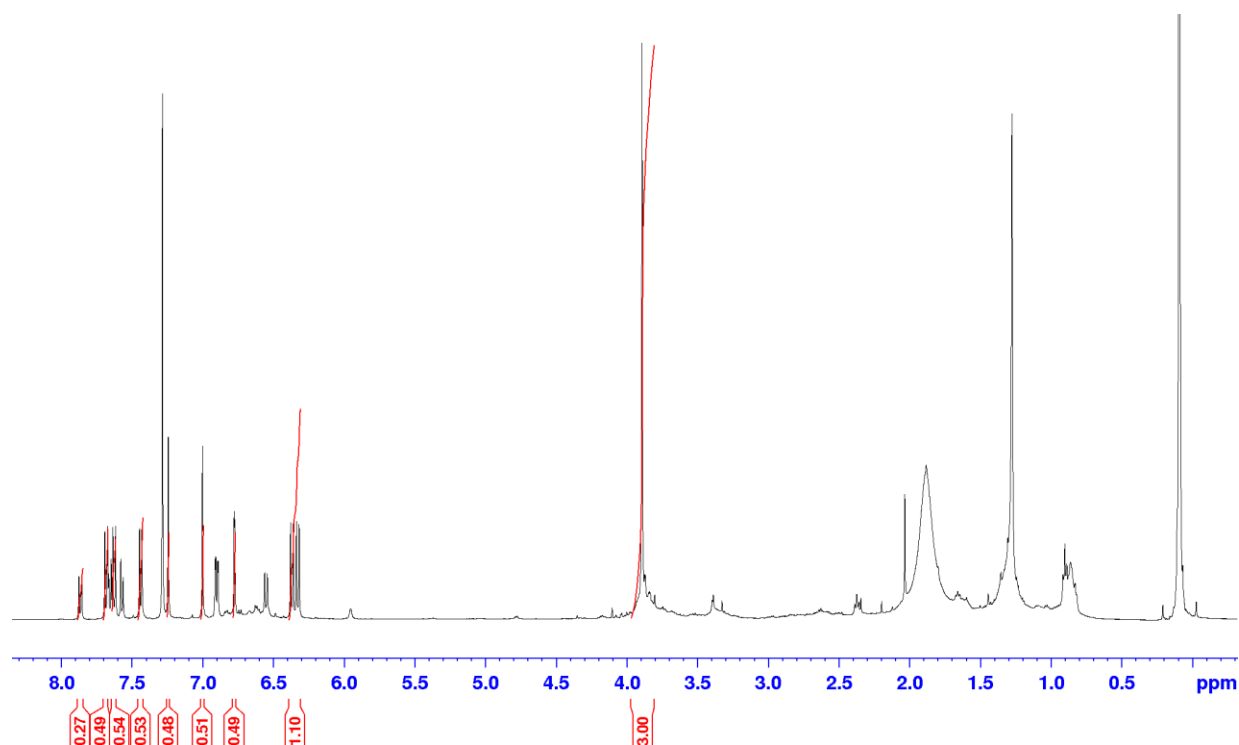
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
305.0456	305.0450	0.6	2.0	14.5	C18 H9 O5	36.4	0.224	79.96	18	9	5
	305.0509	-5.3	-1...	5.5	C11 H13 O10	37.8	1.608	20.04	11	13	10

20190607_Babalwa_BT_I_33CB_p 581 (6.624)

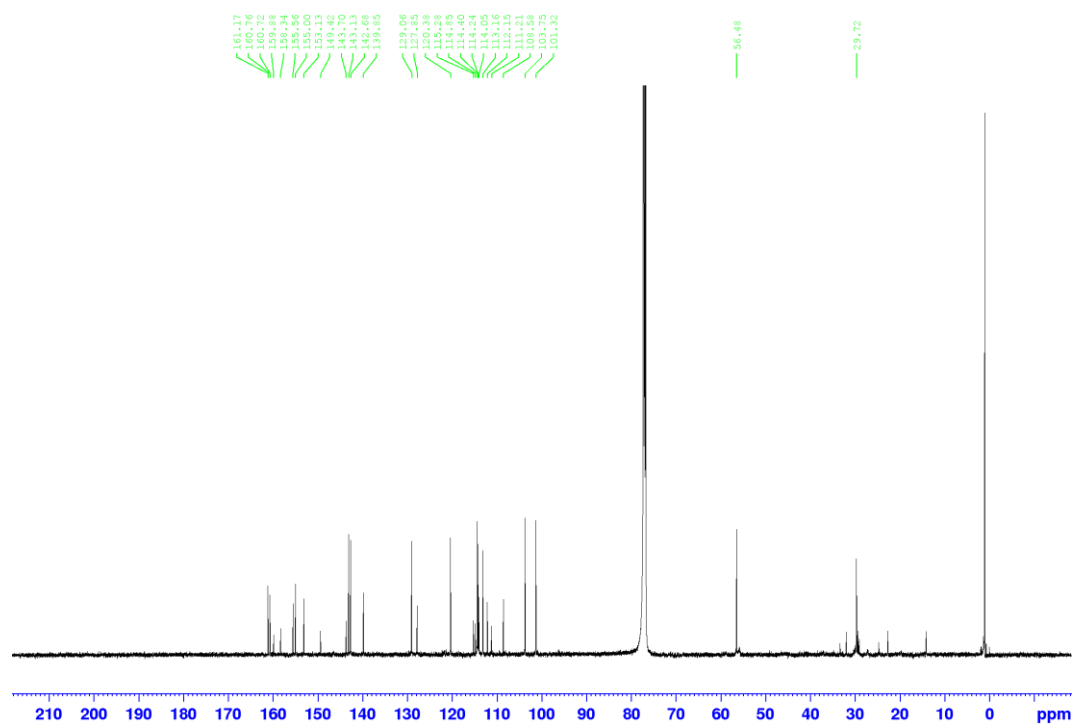
2: TOF MS ES+
2.59e4



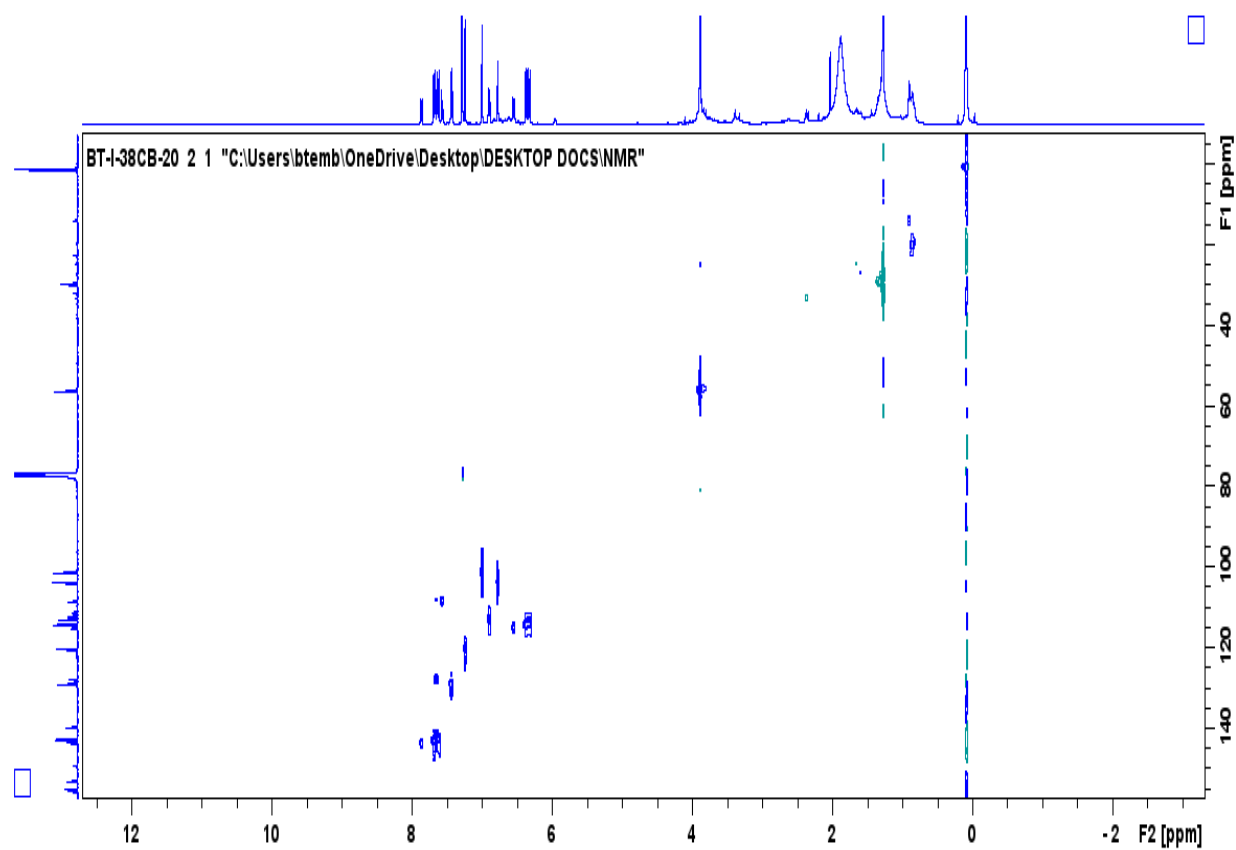
Supplementary data 6: MS/MS spectral data of lasioerin



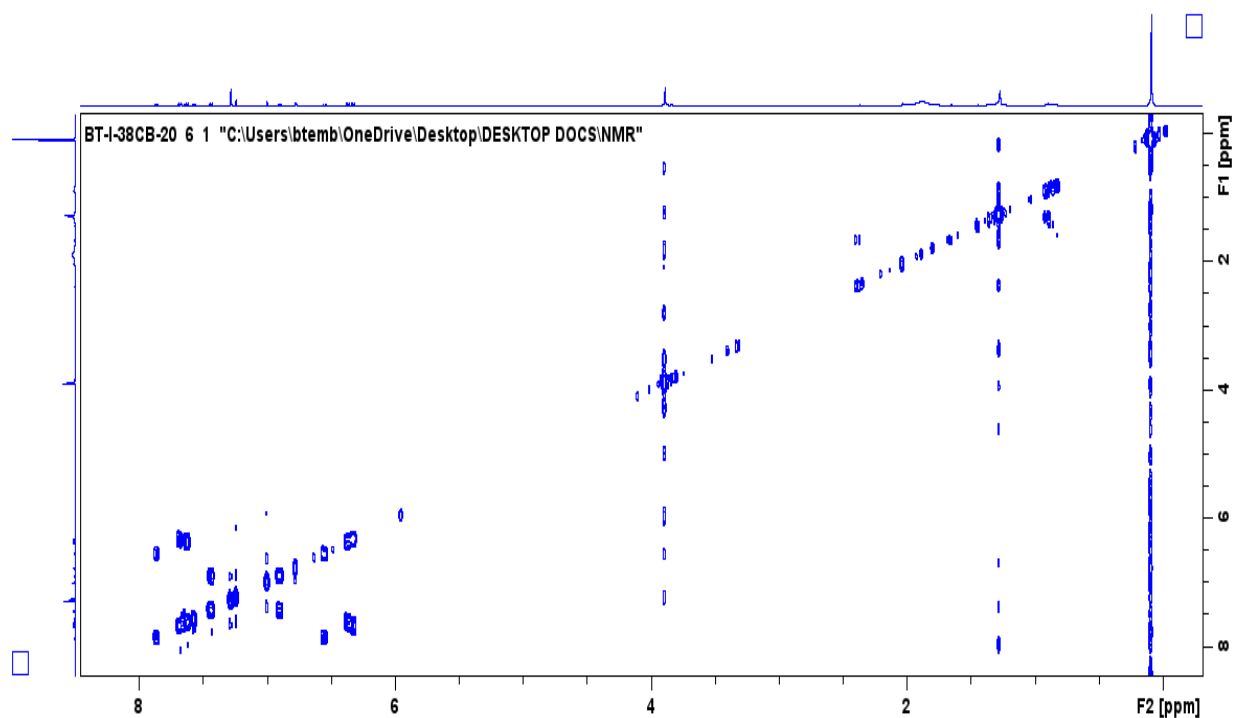
Supplementary data 7: ¹H-NMR Spectra of lasiocephalin in a 2:1 ratio with lasioerin



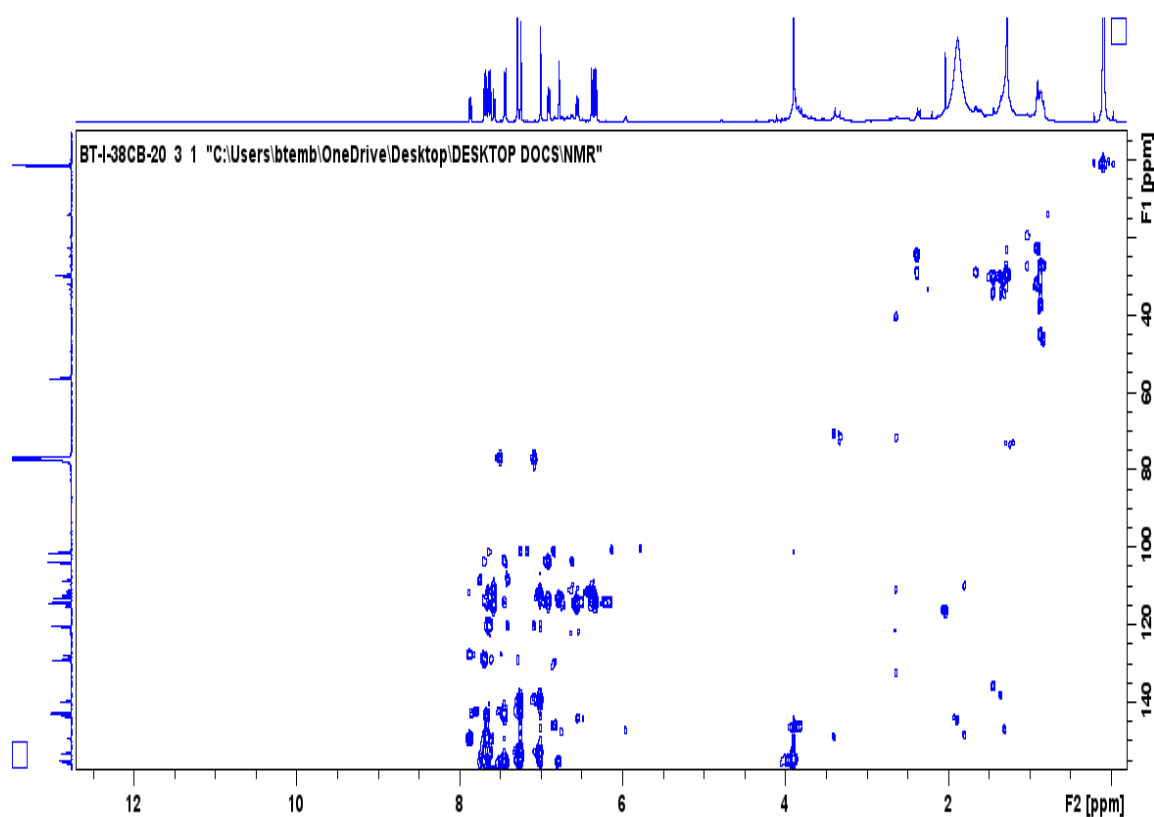
Supplementary data 8: ^{13}C -NMR Spectra of 2:1 ratio with lasioerin



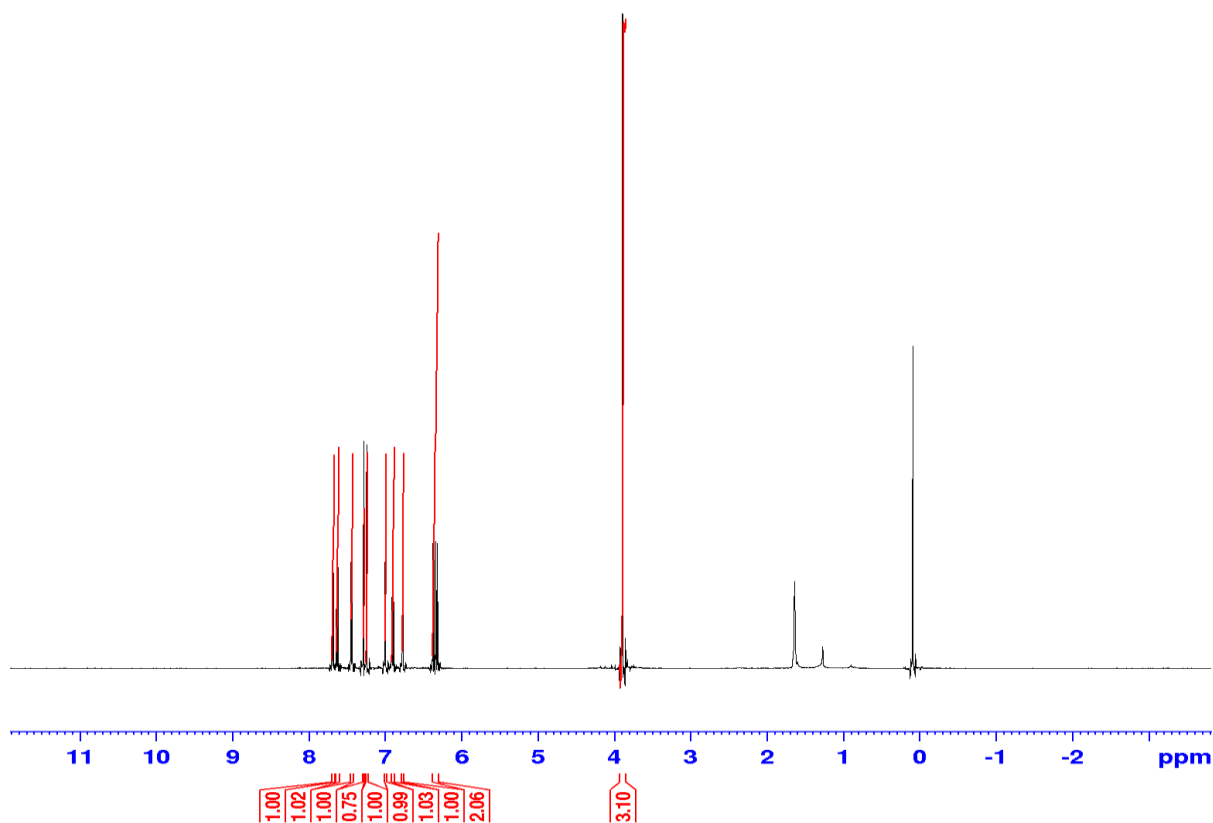
Supplementary data 9: HSQC NMR spectra of lasiocephalin in a 2:1 ratio with lasioerin



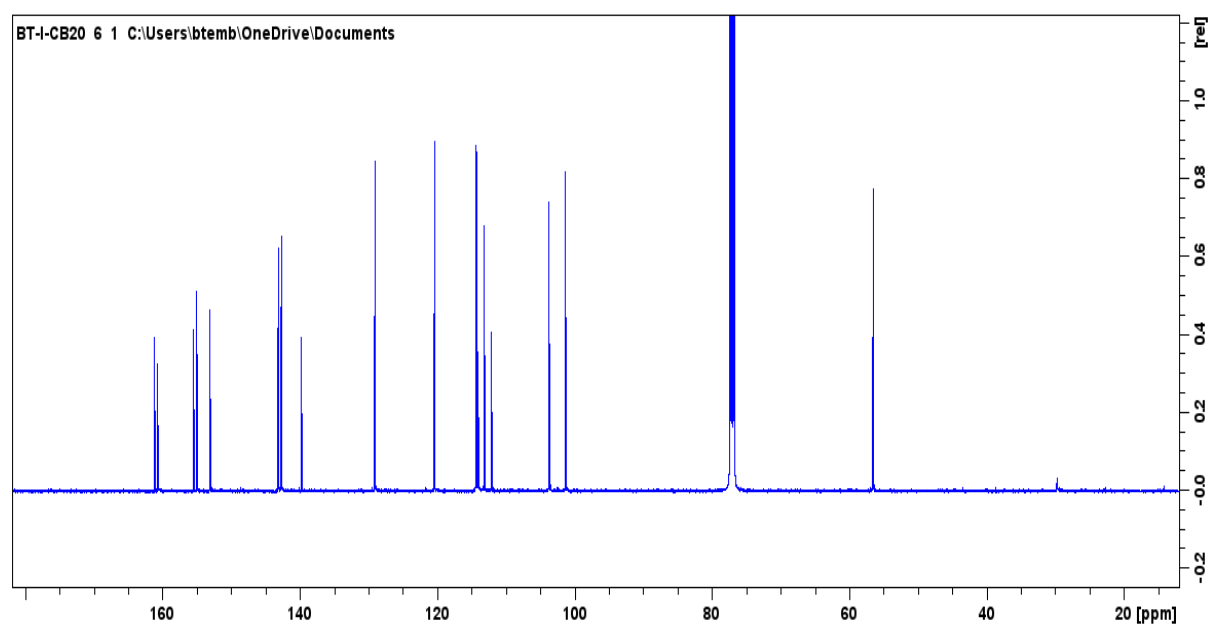
Supplementary data 10: ¹H-¹H COSY NMR spectra of lasiocephalin in a 2:1 ratio with lasioerin



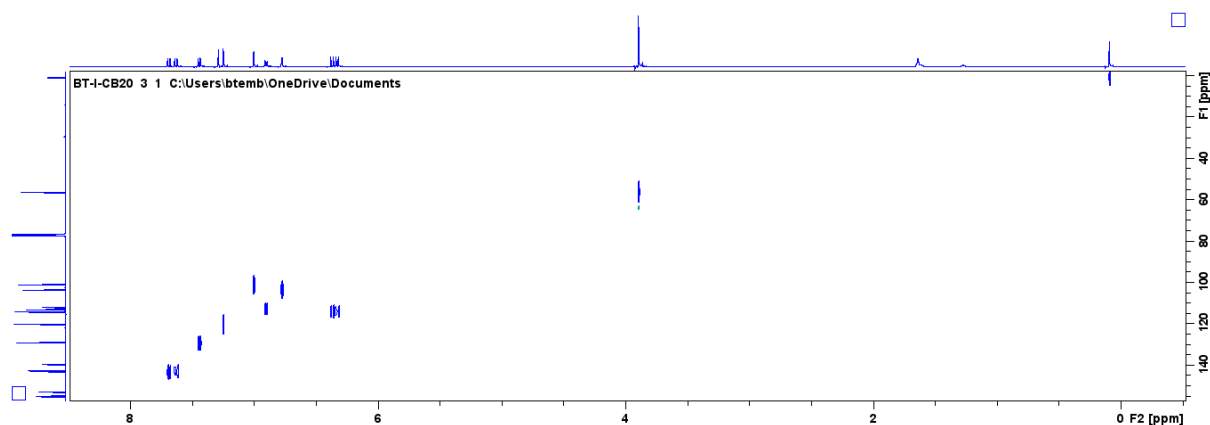
Supplementary data 11: HMBC NMR spectra of lasiocephalin in a 2:1 ratio with lasioerin



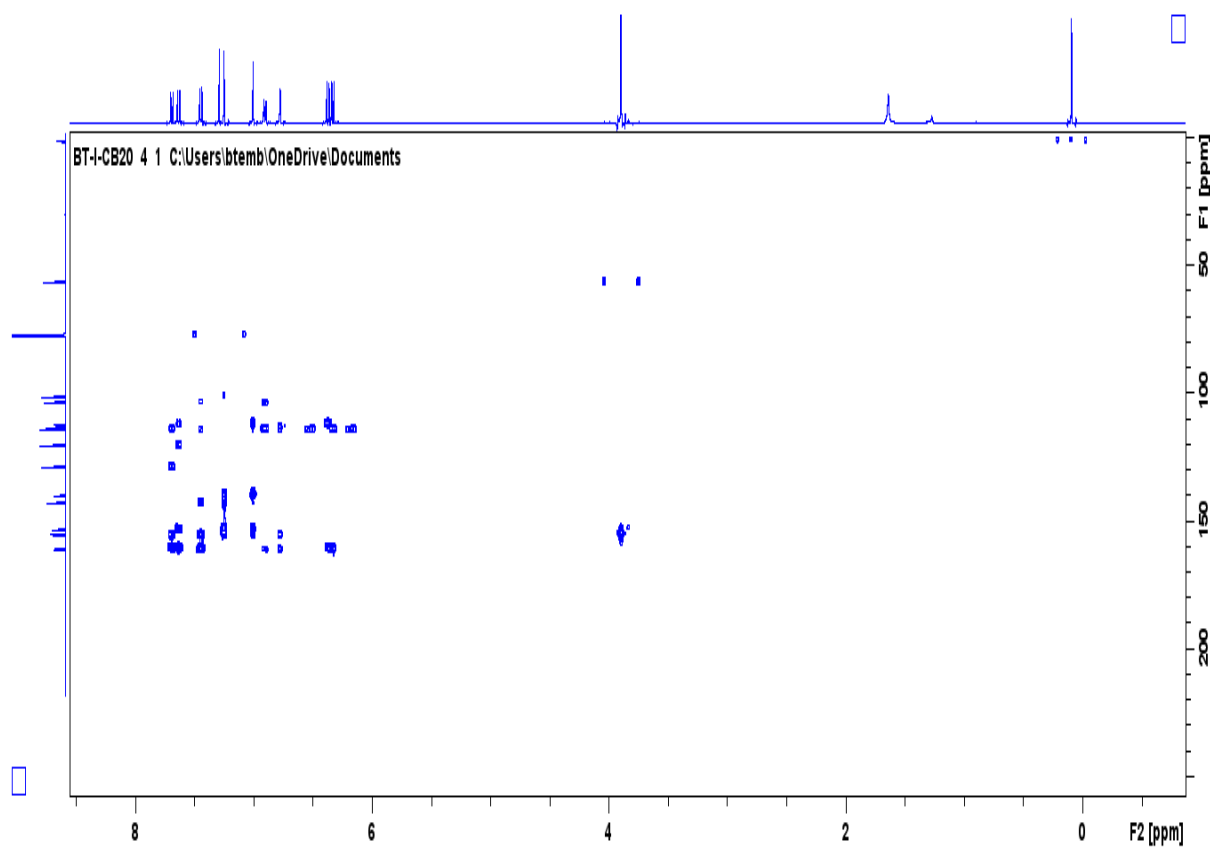
Supplementary data 12: ¹H-NMR spectrum of lasiocephalin



Supplementary data 13: ¹³C-NMR Spectrum of lasiocephalin



Supplementary data 14: HSQC spectrum of lasiocephalin



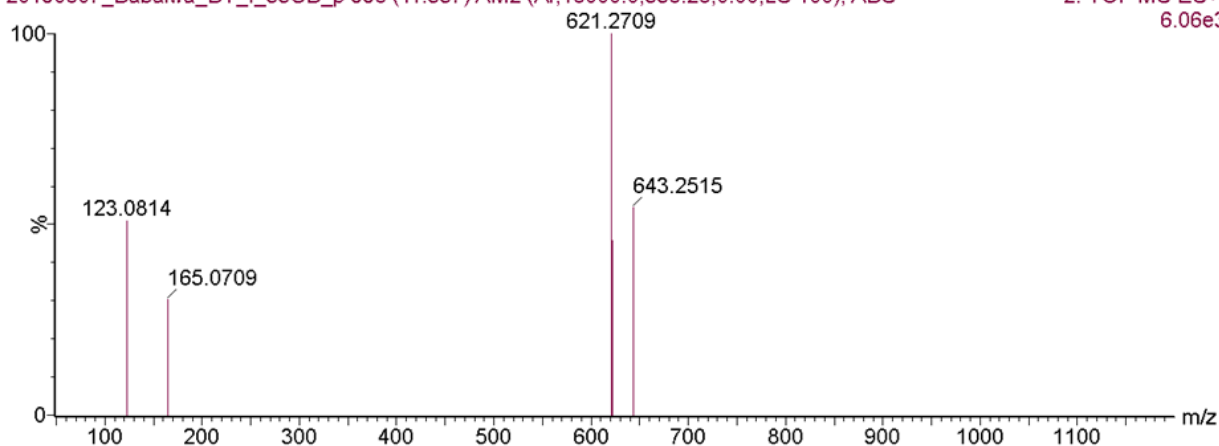
Supplementary data 15: HMBC spectrum of lasiocephalin

Single Mass Analysis
Tolerance = 6.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
183 formula(e) evaluated with 2 results within limits (up to 5 closest results for each mass)
Elements Used:

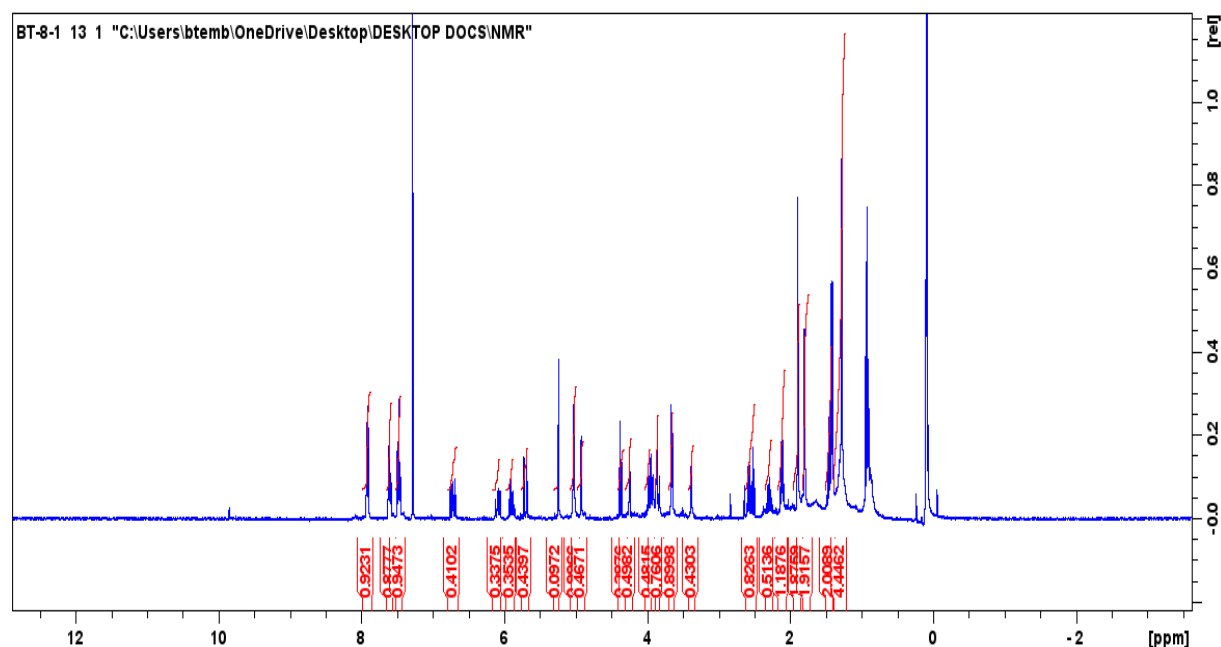
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
621.2701	621.2700	0.1	0.2	15.5	C35 H41 O10	35.9	0.095	90.95	35	41	10
621.2758		-5.7	-9.2	6.5	C28 H45 O15	38.2	2.403	9.05	28	45	15

20190607_Babalwa_BT_I_33CB_p 998 (11.357) AM2 (Ar,18000.0,556.28,0.00,LS 100); ABS

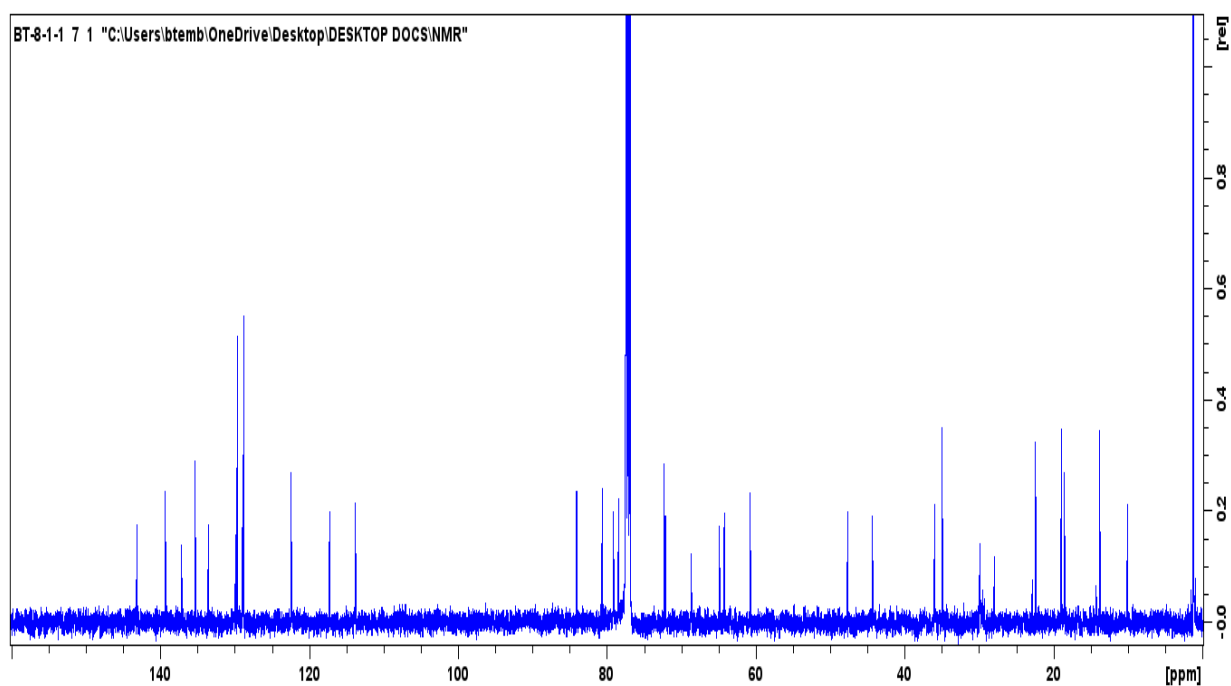
2: TOF MS ES+
6.06e3



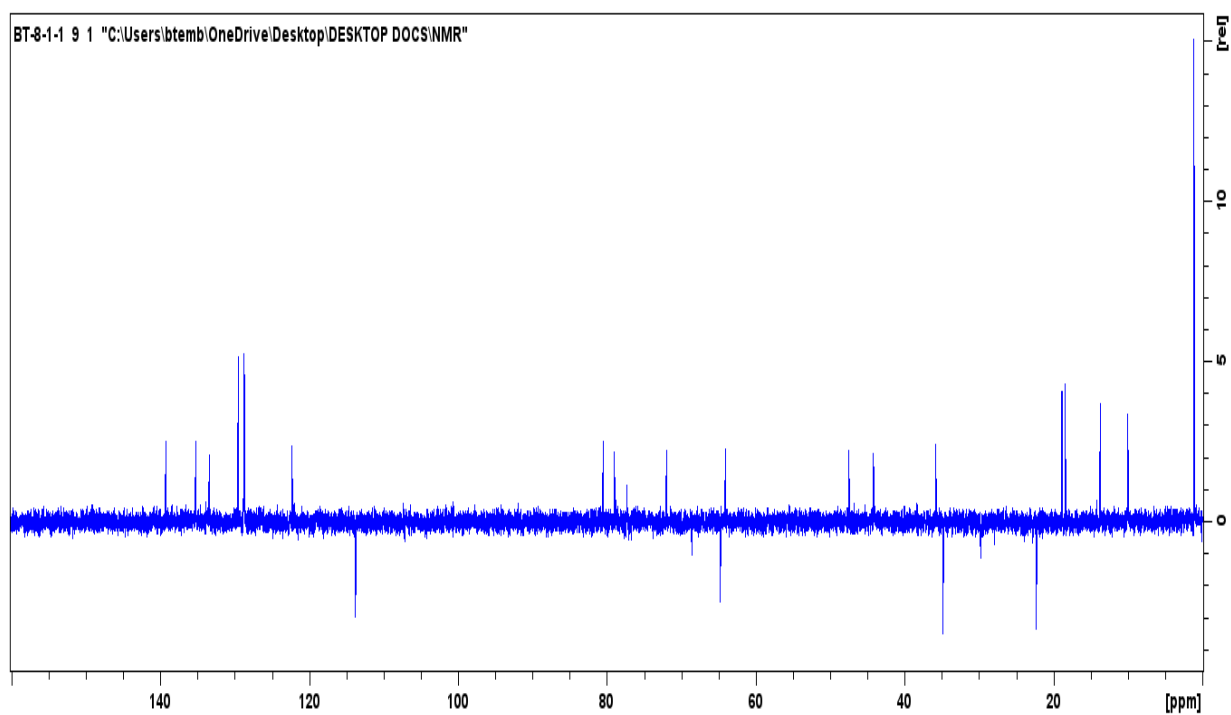
Supplementary data 16: MS/MS spectral data of yuanhuacine A



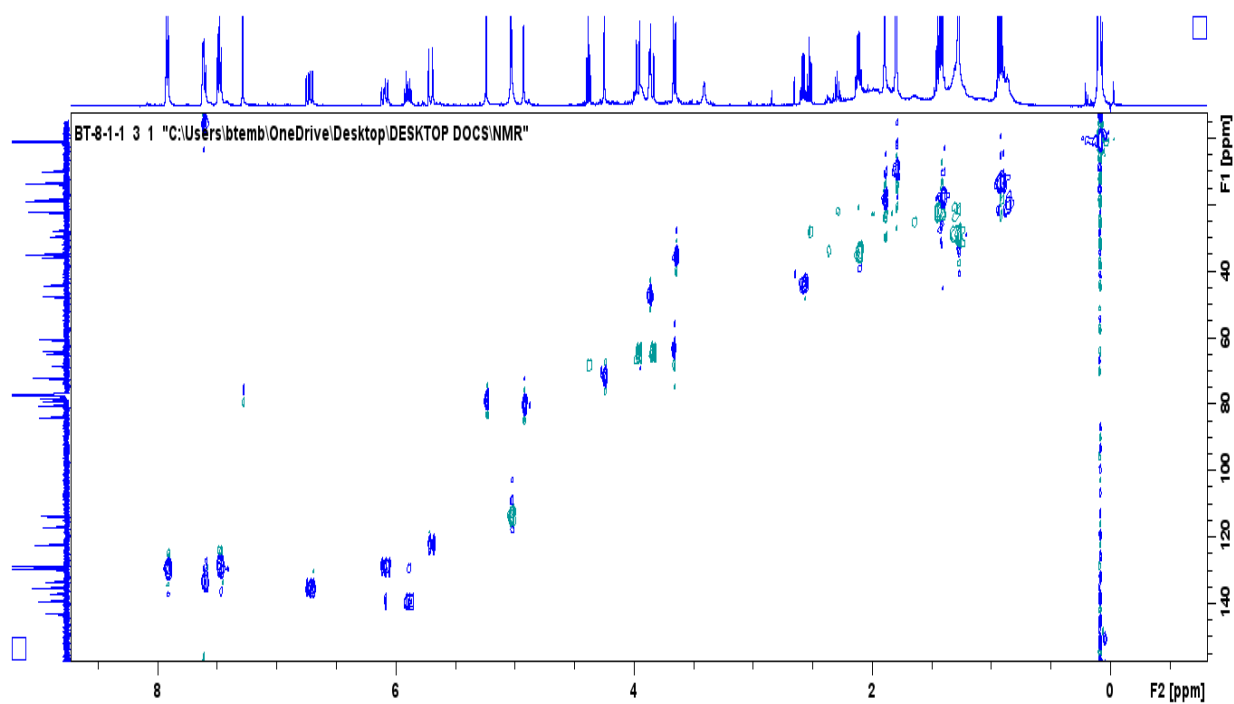
Supplementary data 17: ¹H-NMR spectra of yuanhuacine A



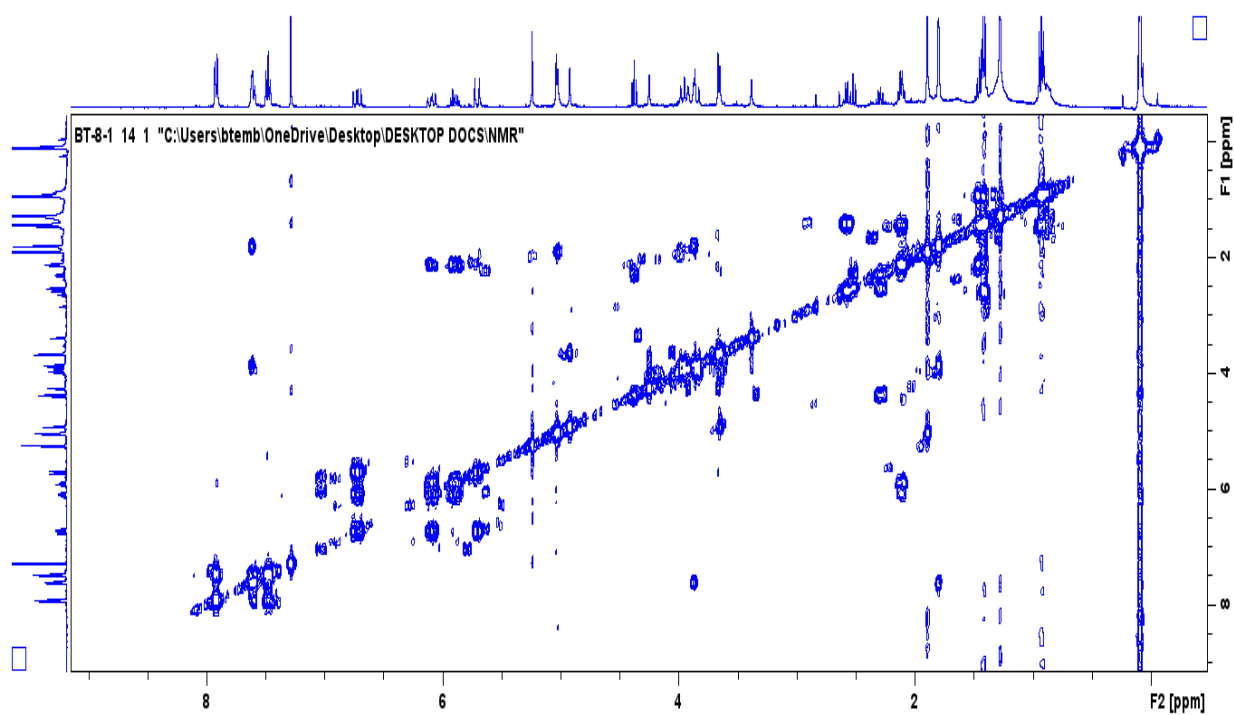
Supplementary data 18: ^{13}C -NMR spectra of yuanhuacine A



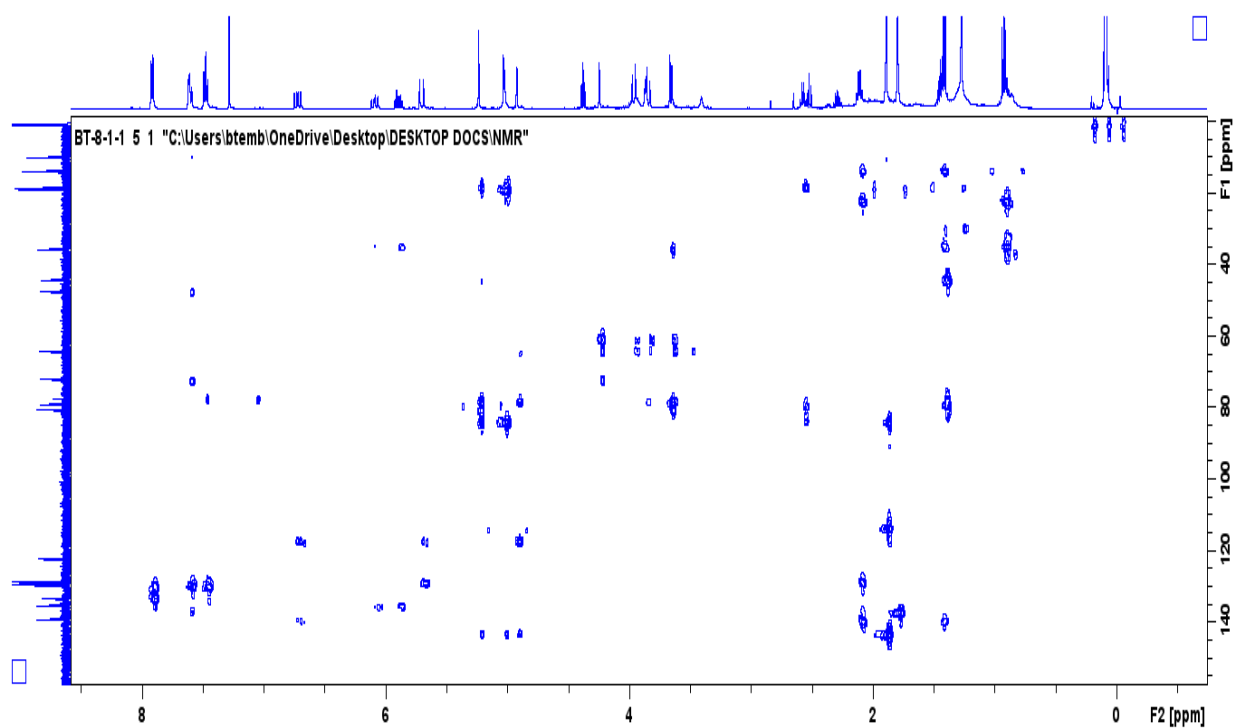
Supplementary data 19: DEBT135 NMR spectra of yuanhuacine A



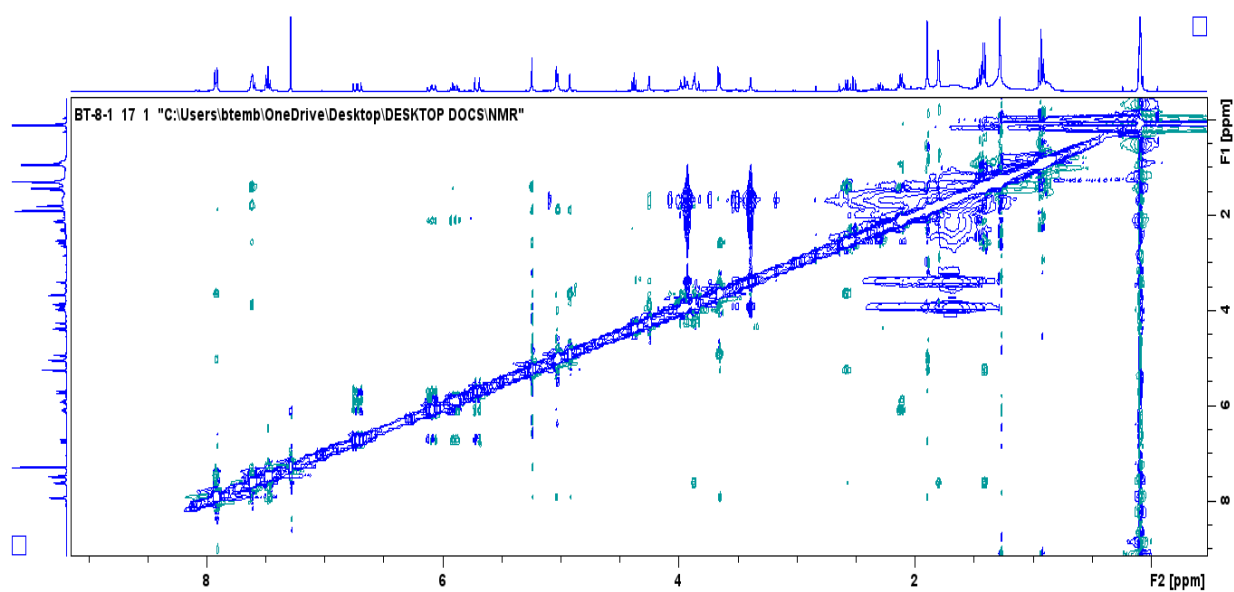
Supplementary data 20: HSQC NMR spectra of yuanhuacine A



Supplementary data 21: ^1H - ^1H COSY NMR spectra of yuanhuacine A



Supplementary data 22: HMBC NMR spectra of yuanhuacine A



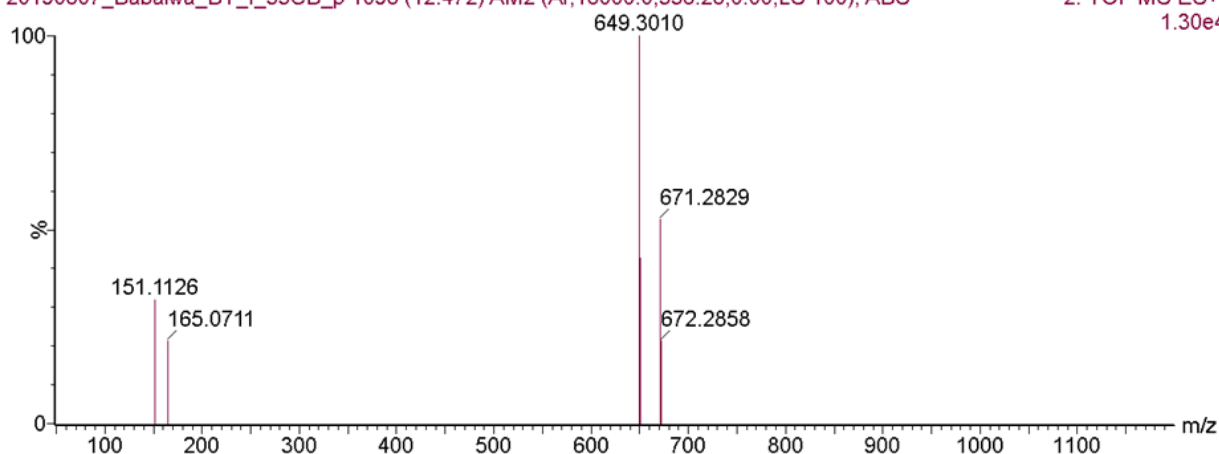
Supplementary data 23: ^1H - ^1H NOESY NMR spectra of yuanhuacine A

Single Mass Analysis
Tolerance = 6.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
199 formula(e) evaluated with 2 results within limits (up to 5 closest results for each mass)
Elements Used:

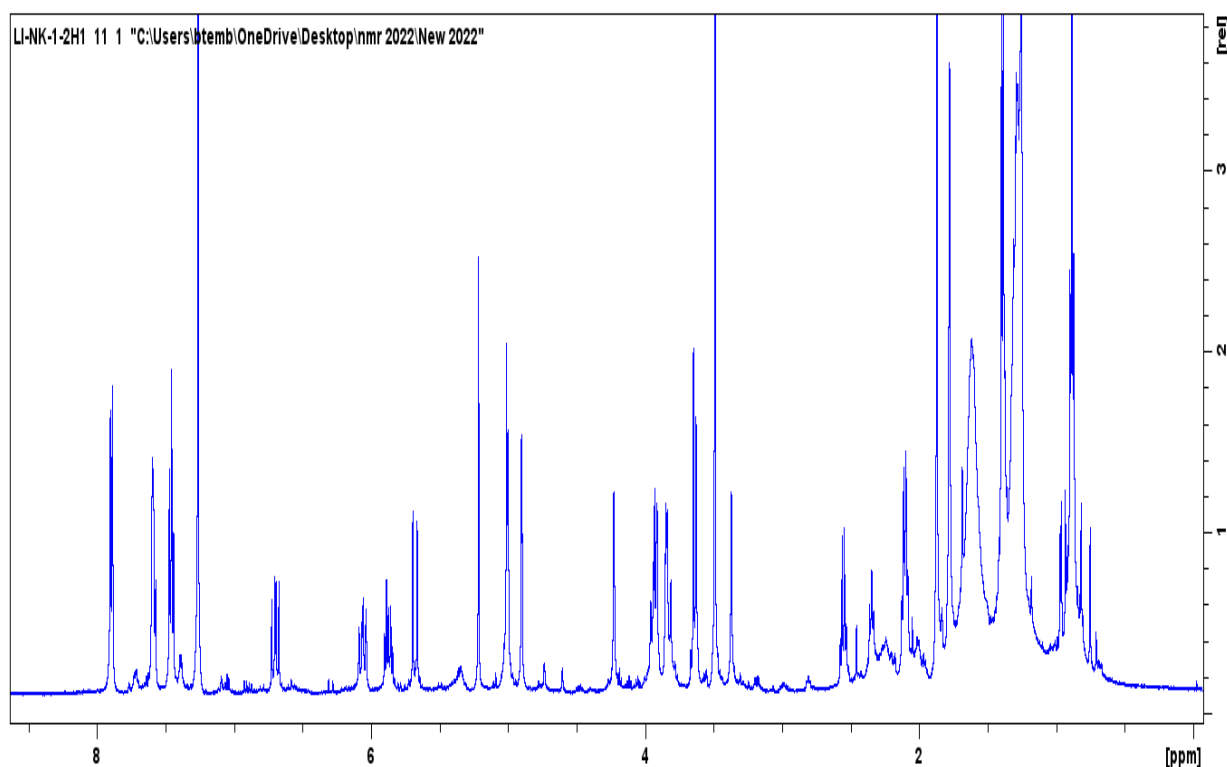
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
649.3016	649.3013	0.3	0.5	15.5	C37 H45 O10	39.6	0.090	91.36	37	45	10
	649.3071	-5.5	-8.5	6.5	C30 H49 O15	41.9	2.449	8.64	30	49	15

20190607_Babalwa_BT_I_33CB_p 1096 (12.472) AM2 (Ar,18000.0,556.28,0.00,LS 100); ABS

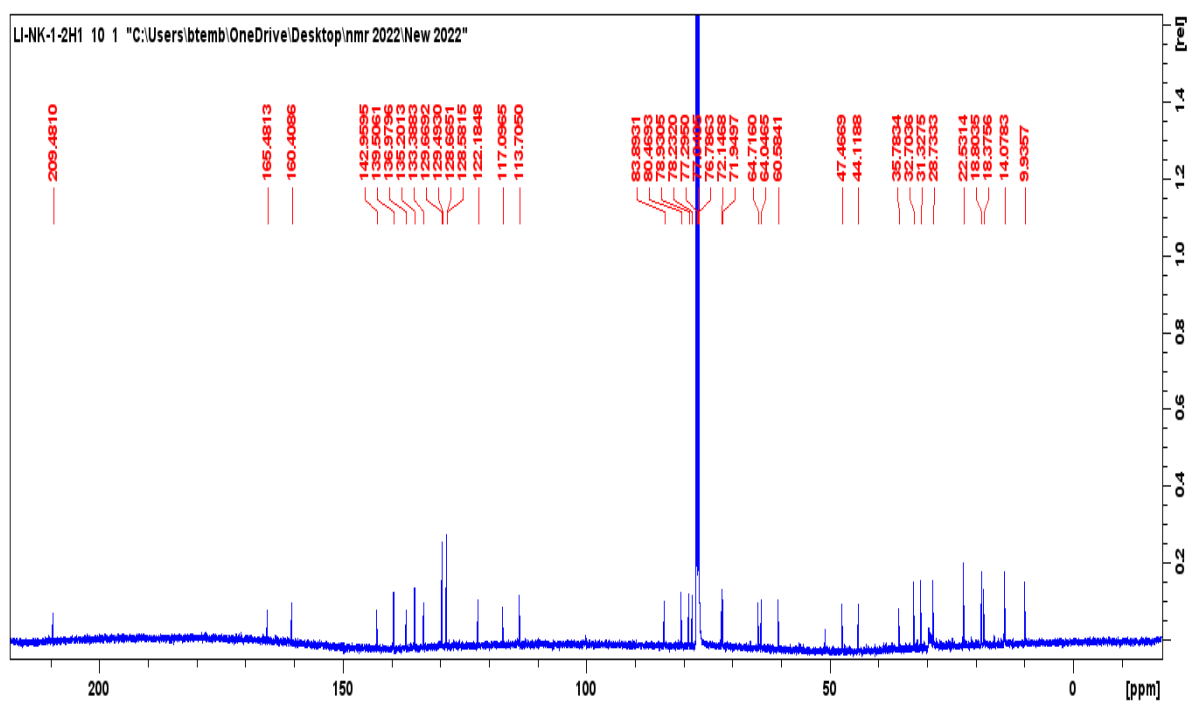
2: TOF MS ES+
1.30e4



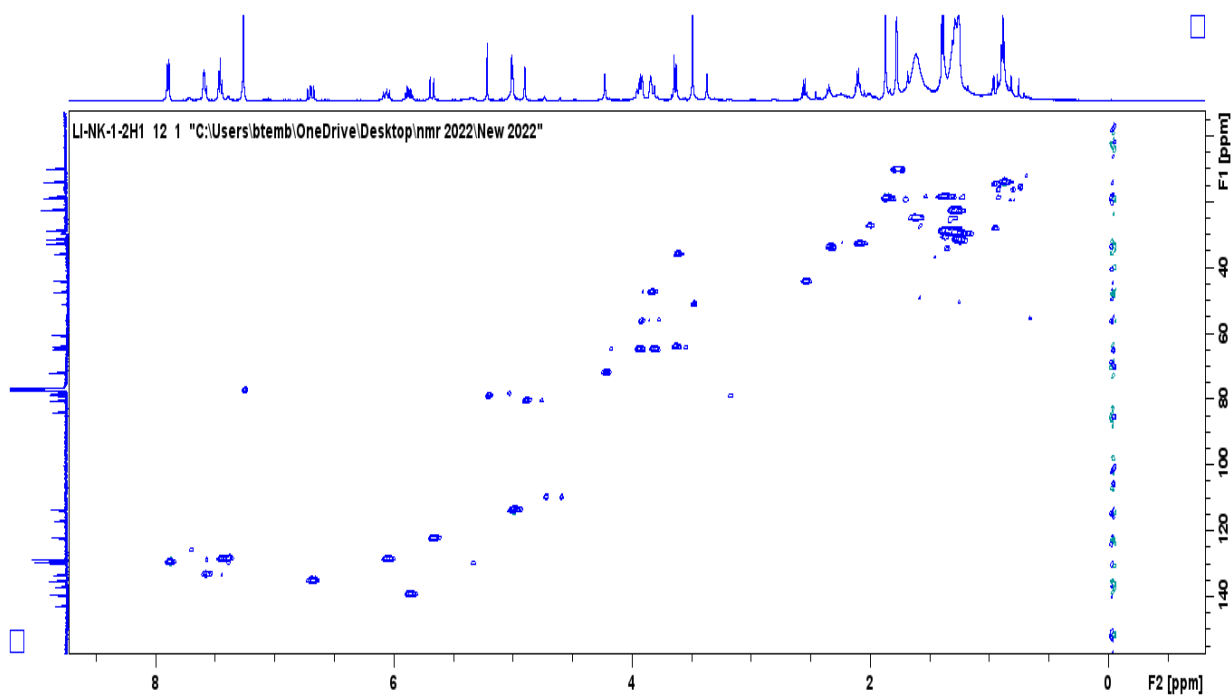
Supplementary data 24: MS/MS Spectral data of yuanhuacine



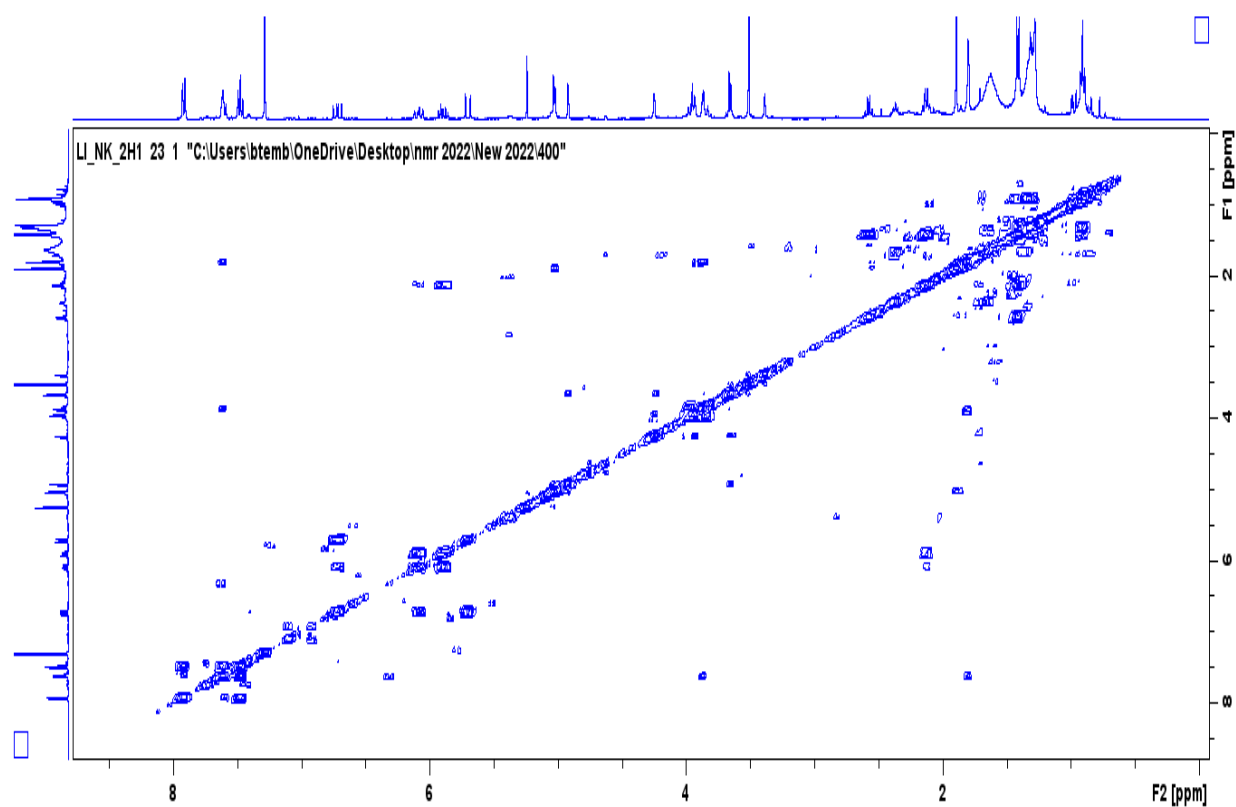
Supplementary data 25: ¹H-NMR spectra of yuanhuacine



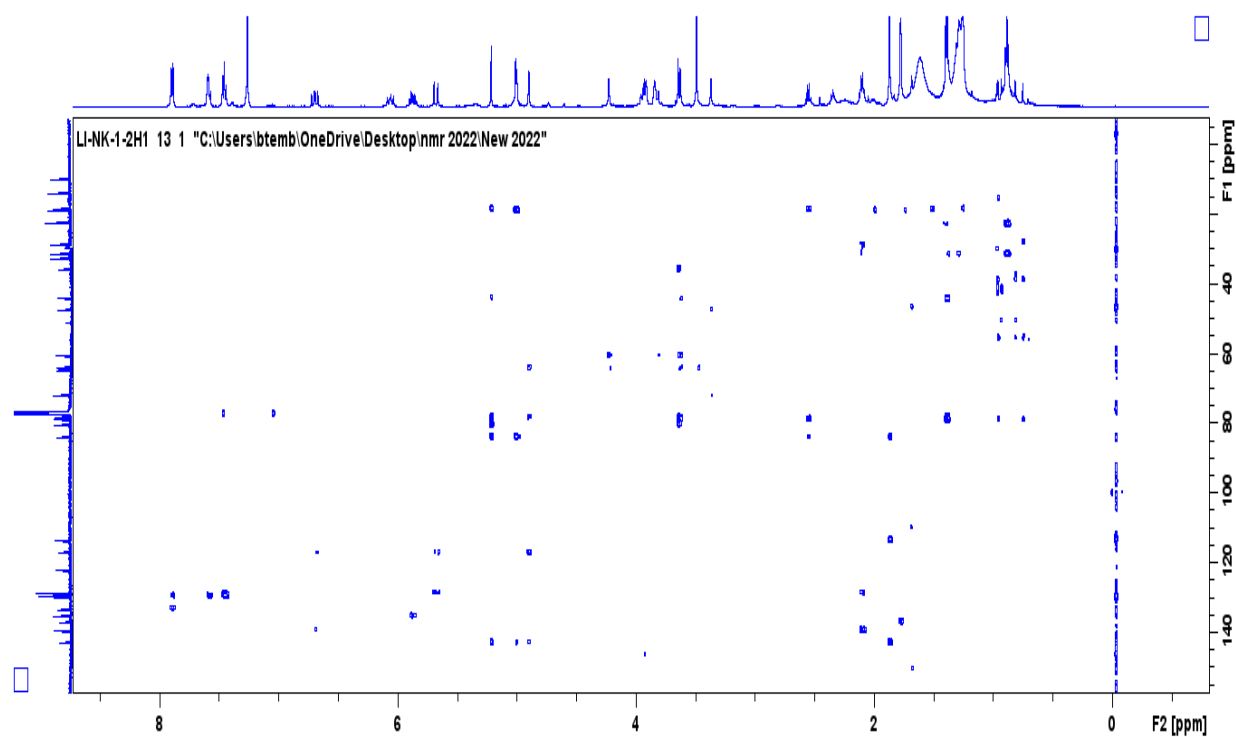
Supplementary data 26: ^{13}C -NMR spectra of yuanhuacine



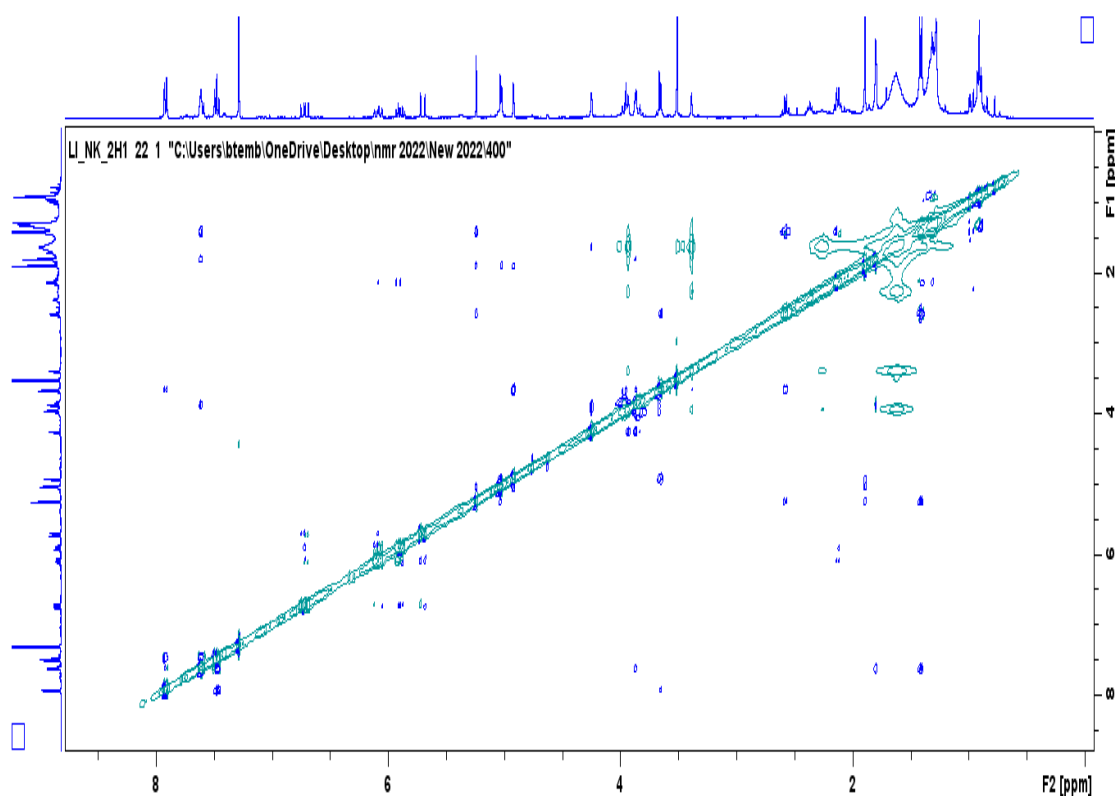
Supplementary data 27: HSQC NMR Spectra of yuanhuacine



Supplementary data 28: ^1H - ^1H COSY NMR spectra of yuanhuacine



Supplementary data 29: HMBC NMR spectra of yuanhuacine



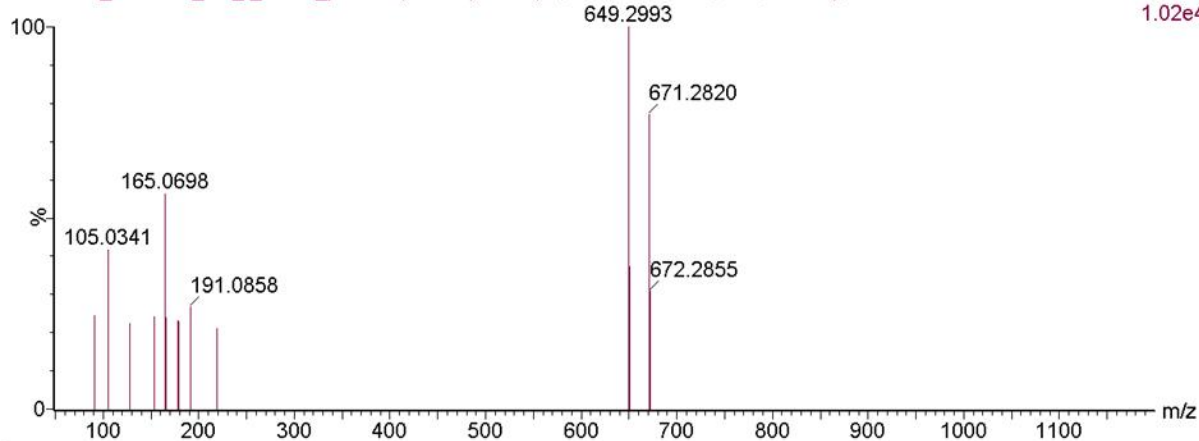
Supplementary data 30: ^1H - ^1H NOESY of yuanhuacine

Single Mass Analysis
Tolerance = 6.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
199 formula(e) evaluated with 3 results within limits (up to 5 closest results for each mass)
Elements Used:

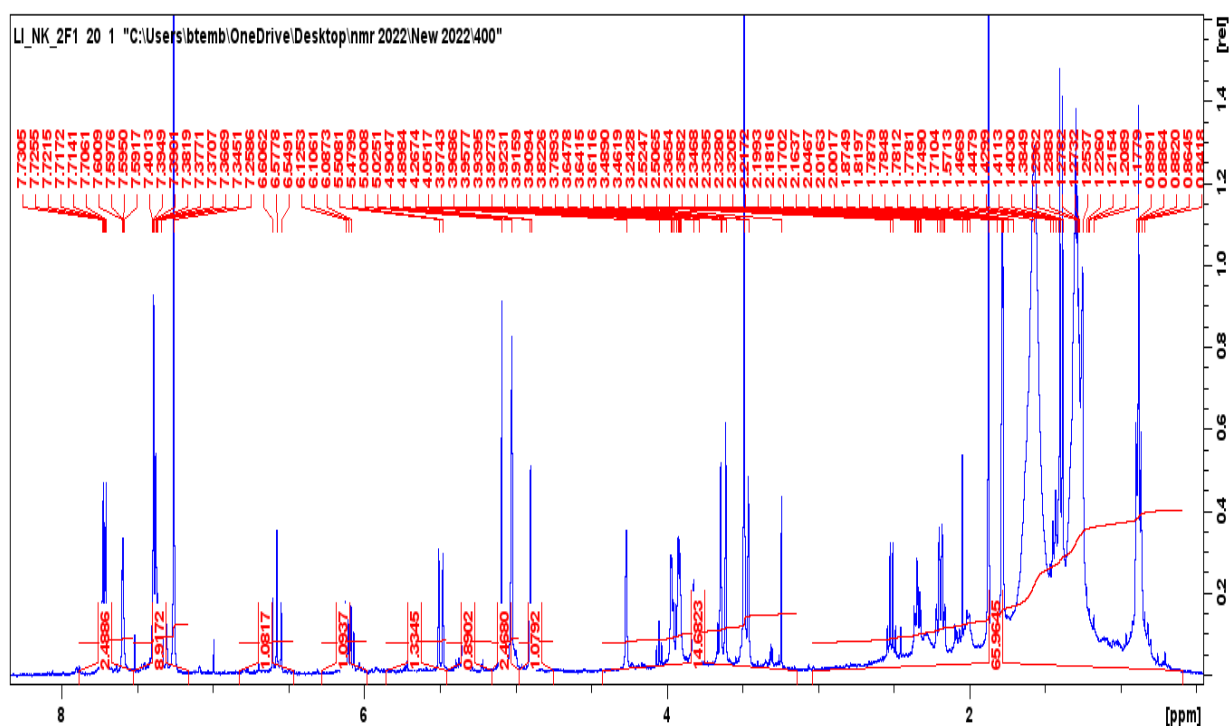
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
649.3013	649.3013	0.0	0.0	15.5	C37 H45 O10	37.7	0.134	87.46	37	45	10
649.3071	-5.8	-8.9	6.5	C30 H49 O15	40.3	2.731	6.51		30	49	15
649.2954	5.9	9.1	24.5	C44 H41 O5	40.4	2.809	6.03		44	41	5

20190607_Babalwa_BT_I_33CB_p 1089 (12.396) AM2 (Ar,18000.0,556.28,0.00,LS 100); ABS

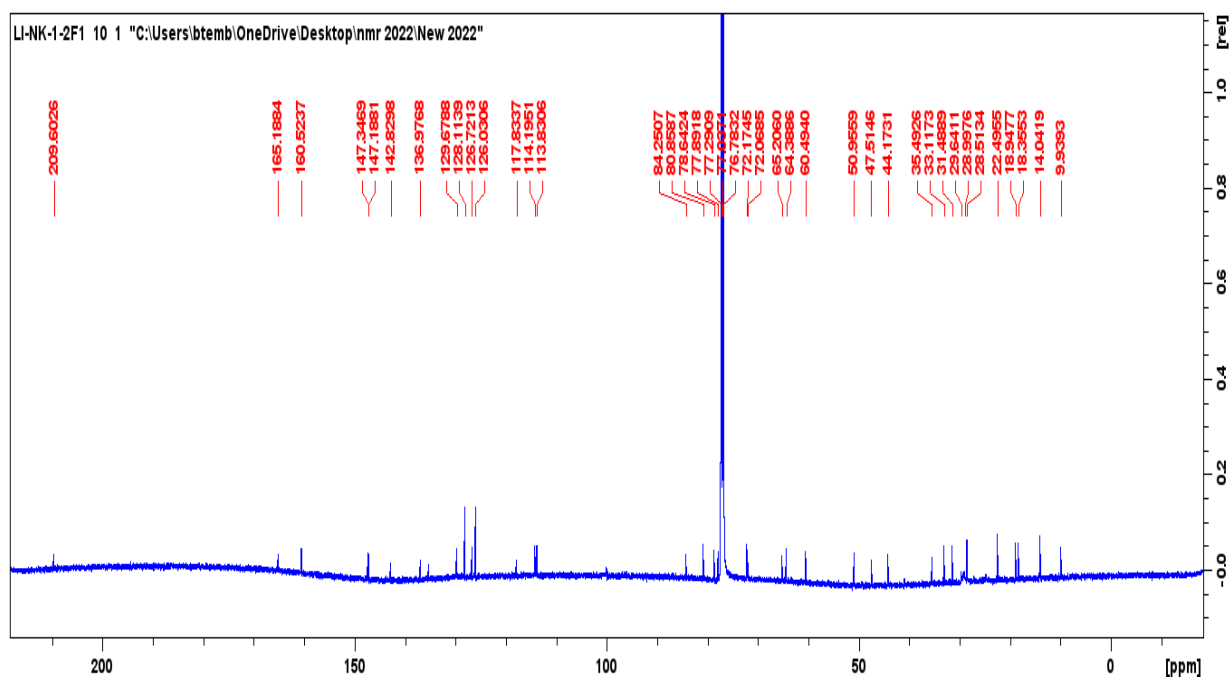
2: TOF MS ES+
1.02e4



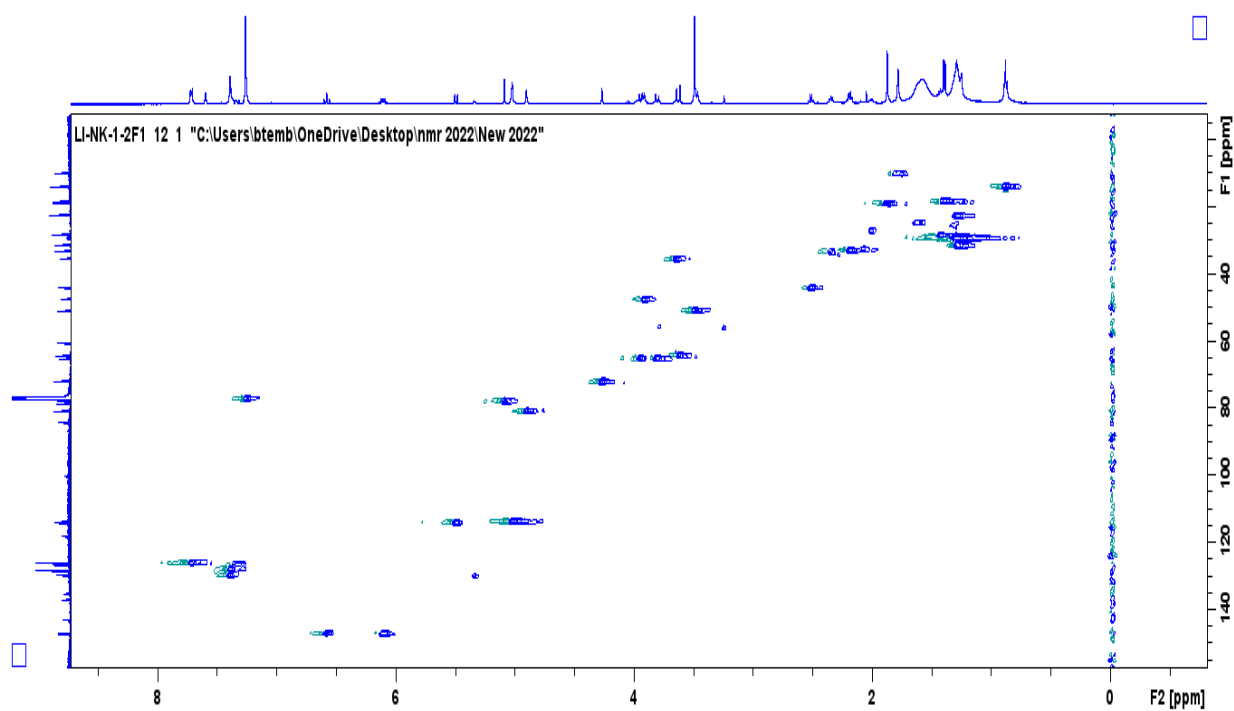
Supplementary data 31: MS/MS spectral data of cis 2''/3'' isomer of gnididin



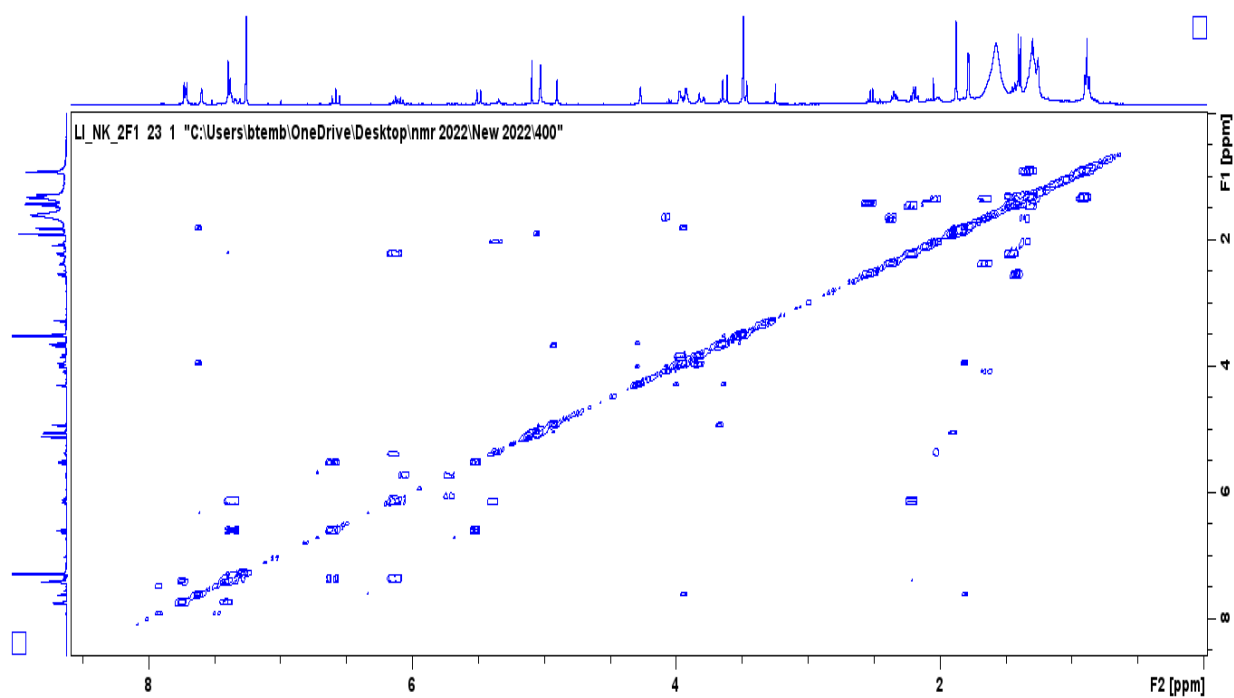
Supplementary data 32: ^1H -NMR spectra of cis 2''/3'' isomer of gnididin



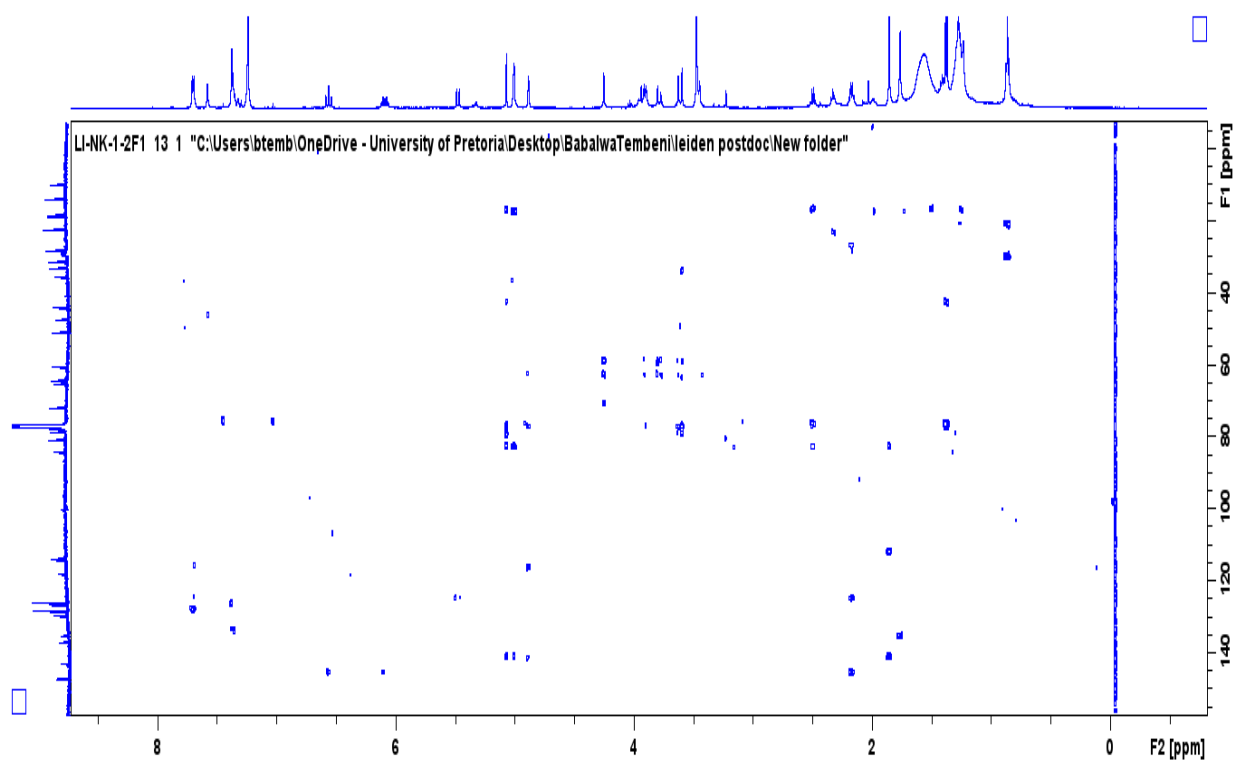
Supplementary data 33: ^{13}C -NMR spectra of cis 2''/3'' isomer of gnididin



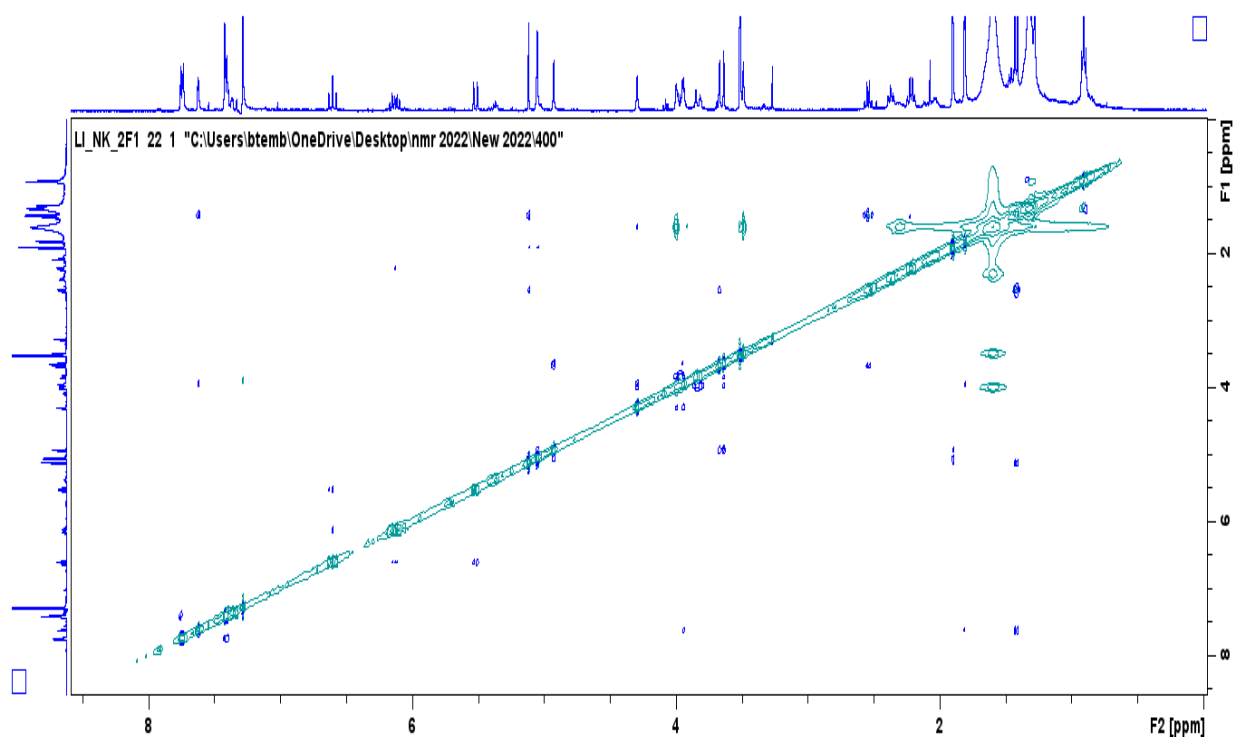
Supplementary data 34: HSQC NMR spectra of cis 2''/3'' isomer of gnididin



Supplementary data 35: ^1H - ^1H COSY NMR spectra of cis 2''/3'' isomer of gnididin



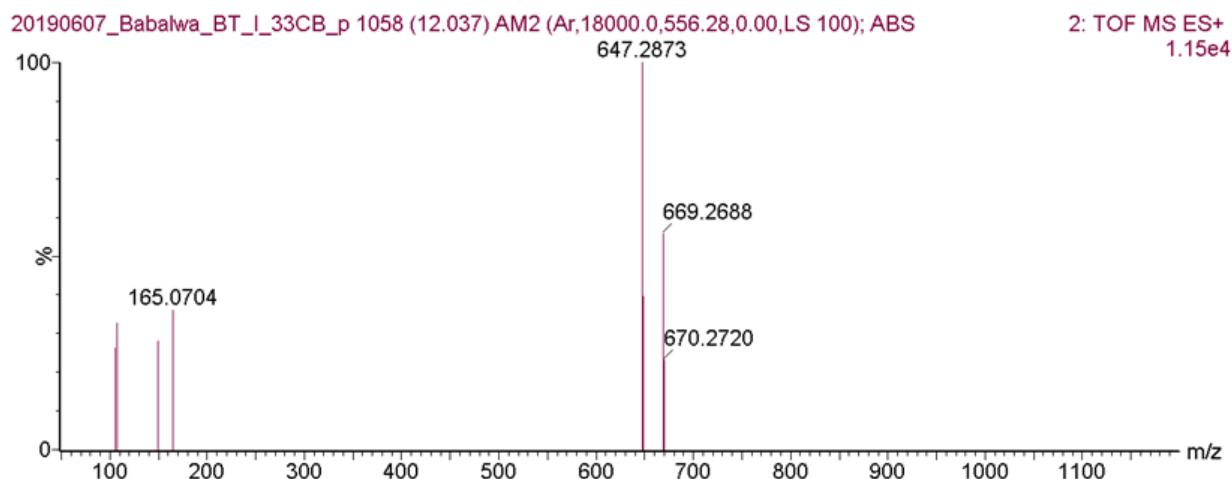
Supplementary data 36: HMBC NMR spectra of cis 2''/3'' isomer of gnididin



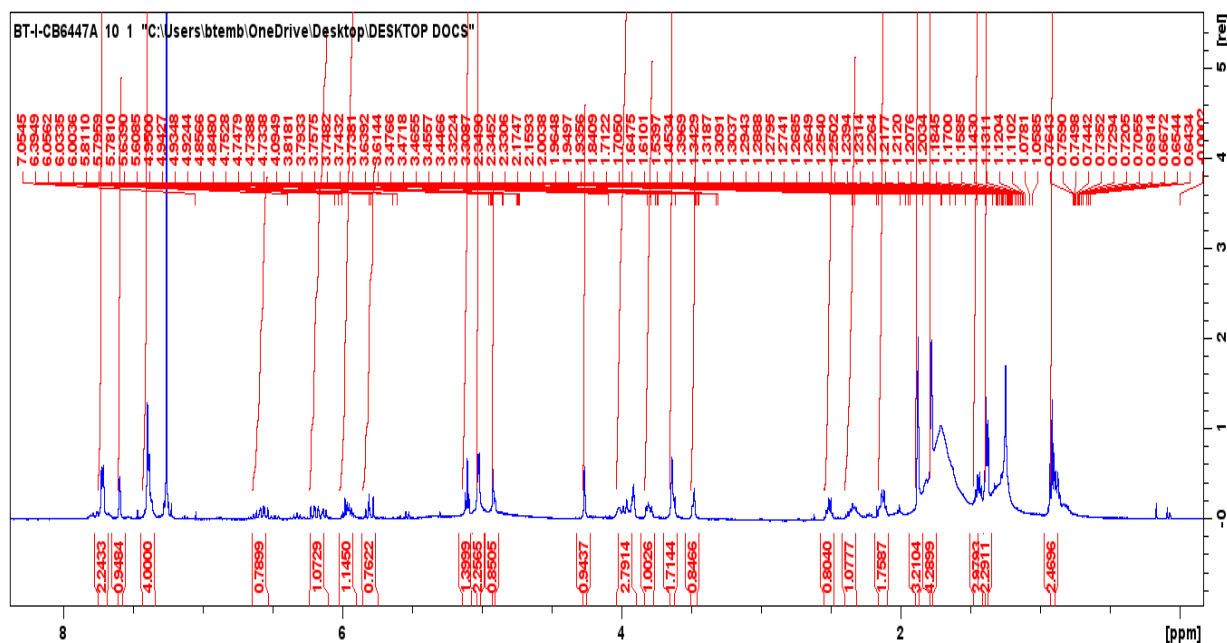
Supplementary data 37: ^1H - ^1H NOESY NMR spectra of cis 2''/3'' isomer of gnididin

Single Mass Analysis
Tolerance = 6.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
205 formula(e) evaluated with 2 results within limits (up to 5 closest results for each mass)
Elements Used:

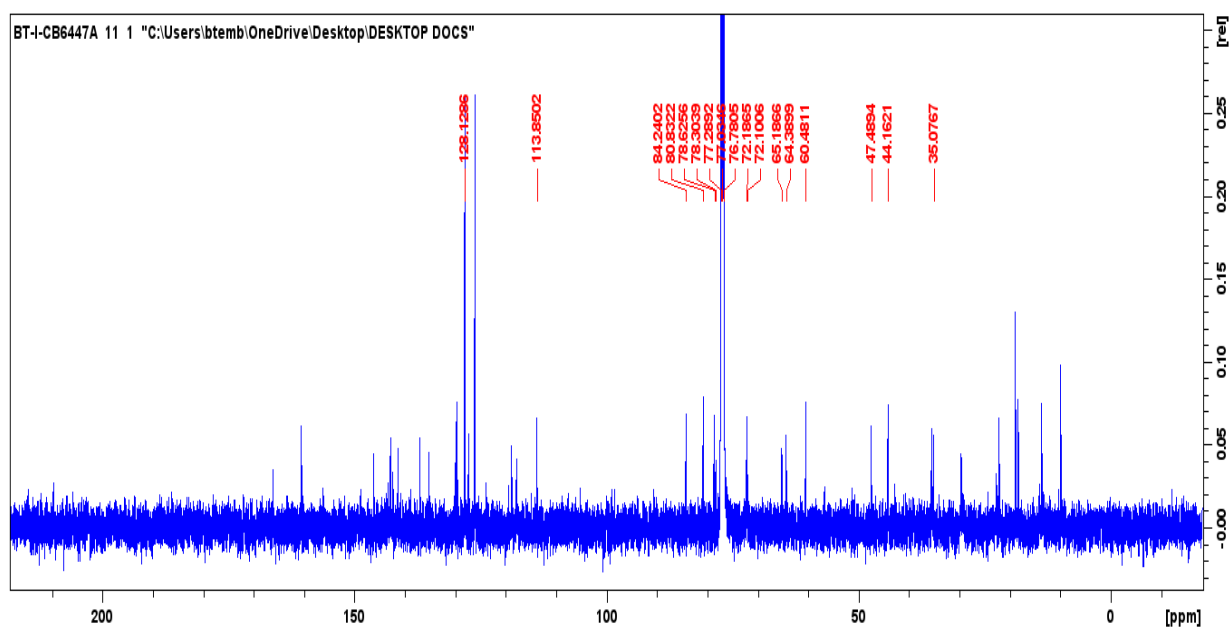
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	O
647.2862	647.2856	0.6	0.9	16.5	C37 H43 O10	39.4	0.372	68.93	37	43	10
	647.2915	-5.3	-8.2	7.5	C30 H47 O15	40.2	1.169	31.07	30	47	15



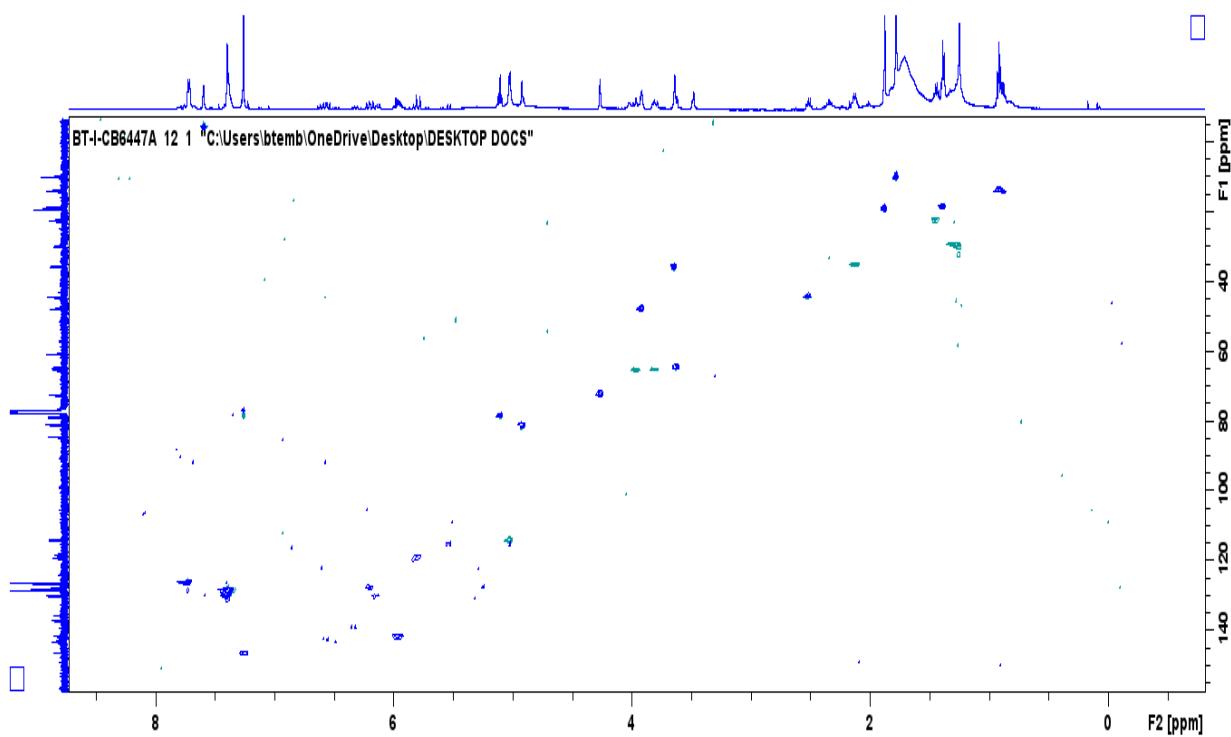
Supplementary data 38: MS/MS spectral data of yuanhuajine



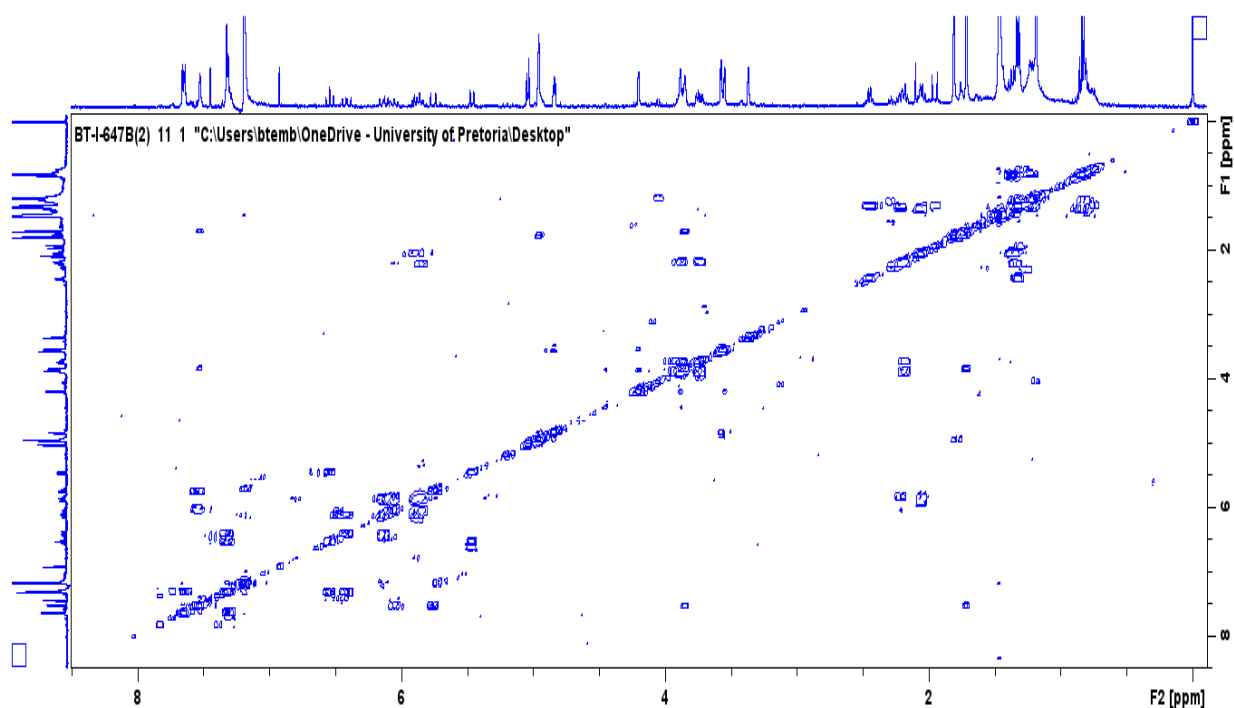
Supplementary data 39: ¹H-NMR spectral data of yuanhuajine



Supplementary data 40: ^{13}C - NMR spectra of yuanhuajine



Supplementary data 41: HSQC NMR spectra of yuanhuajine



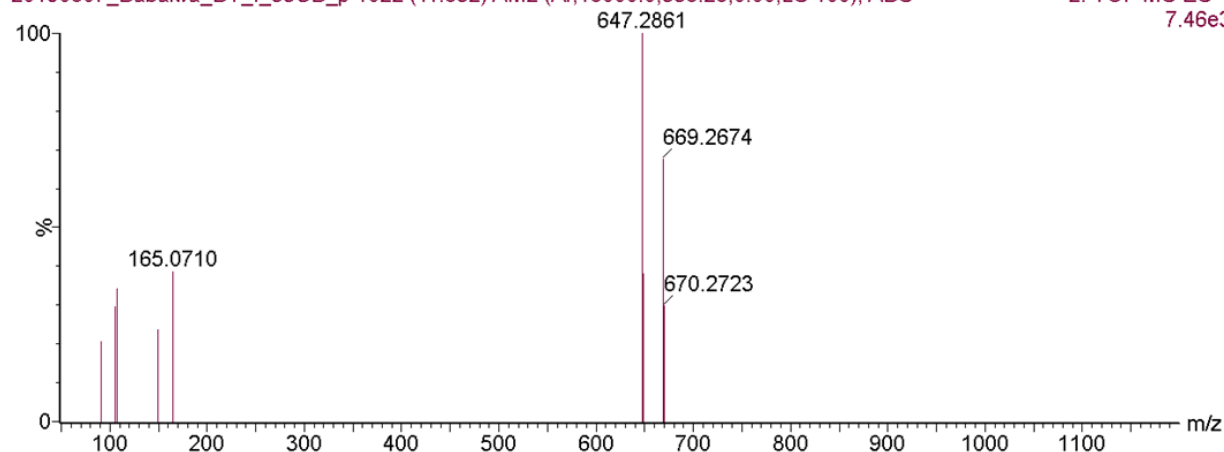
Supplementary data 42: ^1H - ^1H COSY NMR spectra of yuanhuajine

Single Mass Analysis
Tolerance = 6.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3
Monoisotopic Mass, Even Electron Ions
205 formula(e) evaluated with 2 results within limits (up to 5 closest results for each mass)
Elements Used:

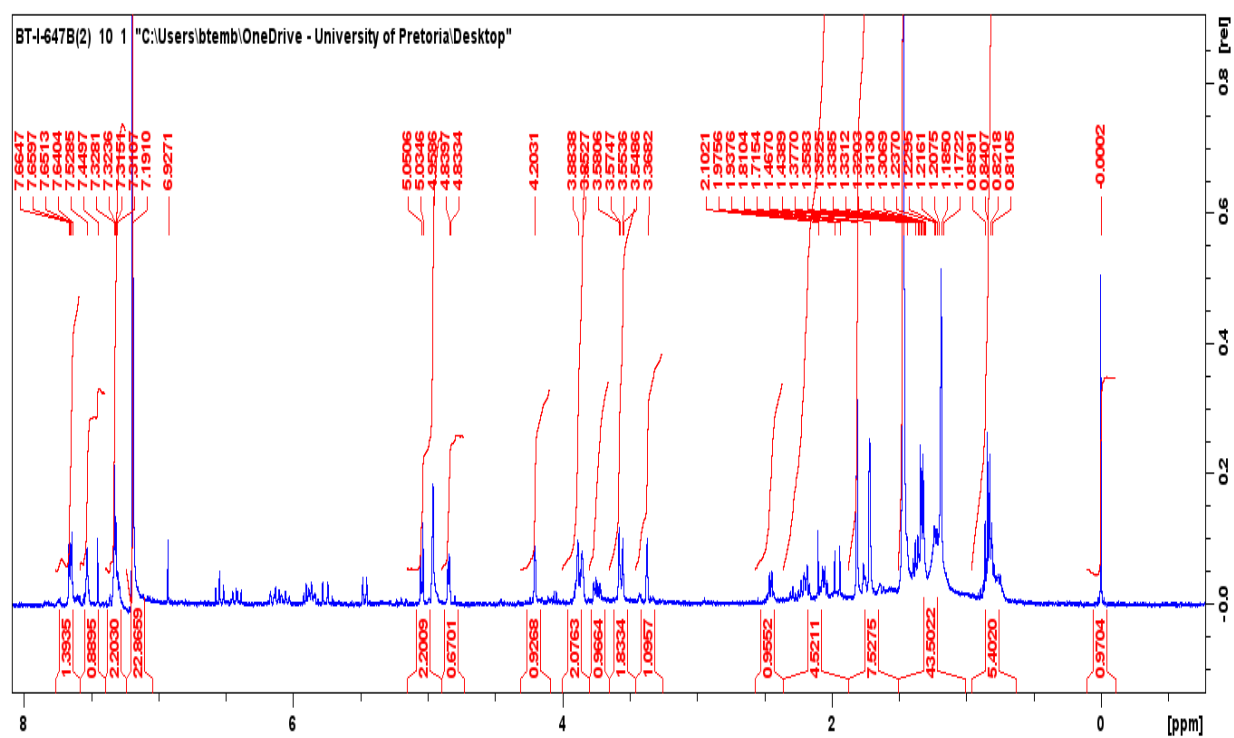
Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit.Conf %	C	H	O
647.2866	647.2856	1.0	1.5	16.5	C ₃₇ H ₄₃ O ₁₀	36.4	0.121	88.59	37	43	10
	647.2915	-4.9	-7.6	7.5	C ₃₀ H ₄₇ O ₁₅	38.5	2.171	11.41	30	47	15

20190607_Babalwa_BT_I_33CB_p 1022 (11.632) AM2 (Ar,18000.0,556.28,0.00,LS 100); ABS

2: TOF MS ES+
7.46e3



Supplementary data 43: MS/MS spectral data of gniditrin



Supplementary data 44: ^1H -NMR spectra of gniditrin